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A BACTERIAL DISEASE OF FOXTAIL (CHAETOCHLOA LUTESCENS)[1]

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Introduction

In a brief note published in March, 1919, the writer called attention to a disease of foxtail common in Arkansas. It was pointed out that the pathogen, a whitish bacillus, was capable of attacking a number of grasses, including most of the common cereals. Since then an intensive study has been made of this disease, with the object of establishing its symptoms and etiology, and in the pursuit of this investigation facts were evolved which clearly showed the necessity of using certain methods in the study of plant pathogens which are not ordinarily in vogue among plant pathologists.

Bacterial Diseases of Gramineae in General

Since the studies here presented indicate that the disease producer has not been previously described it seems desirable, for comparison, to review briefly the bacterial diseases of the *Gramineae*. Smith (I, '20, p. 7)[2] has recently given a list of plant genera which are known to be attacked by one or more bacterial species. In this is included the following grass genera: *Hordeum, Dactylis, Bromus, Zea, Setaria, Andropogon, Avena, Saccharum, Secale, Triticum, Phleum, Poa,* and *Agropyron.* These 13 genera,

[1]Submitted for publication May, 1922.

[2]The bibliography is divided into two parts, the first part dealing with references to bacterial diseases of grasses, designated I, and the second part dealing with morphological and physiological references, designated II.

he also states (p. 4), are attacked by 14 different disease producers. Not having mentioned these by name or by citation to literature, it becomes rather difficult to ascertain the organisms which he included. It is quite possible that 1 or more of these 14 have not as yet appeared in print and are known only to Dr. Smith. Of the host genera which he lists, no reference has been found to a bacterial disease of *Bromus*, and aside from Manns' (I, '09) brief reference to a disease on the blue grasses which, according to the author, appeared to be the same disease as that on oats, no other literature dealing with a bacterial disease of *Poa* has been found. Concerning a bacterial disease of *Phleum* but little more information is available. Manns refers to a bacterial disease of timothy in the same sentence, cited above, in which reference is also made to a disease of *Poa*. In addition to this reference, a blade blight of timothy "apparently bacterial" was reported in Ohio during 1918 and 1919 by the Plant Disease Survey (I, '19, p. 157, and '20, p. 76), and Jones, Johnson, and Reddy (I, '17) mention a bacterial disease of *Phleum pratense*.

Of the bacterial diseases of grasses which are well known or supposedly well known it will perhaps be desirable to characterize briefly each one, noting the similarities and the differences as compared with the foxtail organism. A chronological sequence will be followed.

One of the very first investigators of bacteria in relation to plant disease and perhaps the first to study carefully a bacterial disease of a grass was Prillieux. His account of a rose-red disease of wheat kernels (I, '78) is well known. He made no pure culture studies, so that it is not possible to identify definitely the organism he described. Gentner (I, '20) has recently published an account of a bacterial disease of barley in which he states that the pathogen when inoculated in pure culture into sterilized kernels of barley, wheat, and corn produces a reddish discoloration. He believes that the organism with which he worked, a rod-shaped, red-pigment producer, named by him *Bacillus cerealium*, is the same as Prillieux's organism and that the *Micrococcus* which Prillieux found associated with discolored wheat kernels represents the spore stage of *B. cerealium*. The fact that this organism produces a red pigment on various media and is a spore producer, an anomaly among plant pathogens, would immediately distinguish it from the foxtail organism.

In 1887 Burrill described a disease of broom-corn and sorghum which he attributed to bacteria. Two years later, Kellerman and Swingle (I, '89), under the name of "sorghum blight," published an account of a disease which they considered the same as Burrill's; they also accepted and seemingly substantiated his work on the pathogenicity of the organism which he had named *Bacillus Sorghi*. Several other investigators before and after Burrill's time have worked on red spots of sorghum, but to this day we have little exact information on the disease. Thus Palmerie and Comes in 1883 decided that red discolorations of sorghum were due to yeasts and bacteria; Radais in 1899 concluded that sorghum blight was due to yeasts; and Busse (I, '05), in a rather extensive paper, showed that various causes can produce reddening of sorghum. Concerning any bacterial disease of sorghum, Busse concluded that there was no specific bacterial pathogen involved, that the bacteria which have been found within the tissues were merely facultative parasites which entered by means of injuries induced by insects and other agents and that *Bacillus Sorghi* was an organism of this sort. The writer does not intend to go extensively into this disease, or rather group of diseases, at this time; suffice it to say that as far as the written descriptions of *B. Sorghi* are concerned the organism cannot be identified. While apparently accepting Burrill's organism in an early publication (Smith and Hedges, I, '05) Smith handles it with great caution in other publications (I, '05, p. 66, p. 92, pl. 20 [opposite p. 150]; '11, p. 62 and 63), not definitely rejecting it but substituting another name, ***Bacterium Andropogoni***. Smith's complete account of *B. Andropogoni* follows: "It is non-sporiferous, polar-flagellate (1–3), and white on culture media, forming small circular colonies on agar-poured plates. It is aerobic, non-liquefying, non-reducing (nitrates)." Manifestly this preliminary description also is so incomplete that it is impossible to make any adequate comparison with an unidentified organism. We may note, however, that the foxtail organism possesses similar characters with the exception that it has but a single polar flagellum and is a marked nitrate reducer. The writer's preliminary studies on a bacterial disease of sorghum present in Arkansas indicates that the pathogen there concerned is different from the foxtail organism.

Following the description of a bacterial disease of sorghum Burrill (I, '89) described a bacterial disease of field corn. For

the present I may merely refer to my descriptions of a bacterial disease of field corn (I, '19[a], '21) and to note, as will be fully discussed in another publication, that Burrill's organism is either not identifiable or is not related to disease production. The pathogen is quite unlike the foxtail organism.

In 1893 Cobb described a bacterial disease of sugar cane. The disease is so thoroughly described by Smith (I, '14, pp. 3–71) that there is no necessity for discussion of it here. We may note, however, that *Pseudomonas vascularum,* a yellow rod, is typically a vascular disease producer. These characters alone are sufficient to distinguish it from the foxtail organism.

A bacterial disease of sweet corn was first described by Stewart (I, '97); this, like the sugar cane disease, is also a vascular parasite, is yellow in color, and according to recent investigations (McCulloch I, '18), is non-motile. All these features, as well as others, indicate that it is quite distinct from the foxtail pathogen.

Another non-motile, yellow parasite is *Aplanobacter Rathayi.* It was first described by Rathay in 1899 as attacking orchard grass near Vienna. According to Smith (I, '14, p. 155), who gives a good account of this disease, it is also present in Denmark. Descriptions indicate that this also is distinct from the motile, white, foxtail organism.

In 1901 Guffroy described knots on rhizomes of *Arrhenatherum elatius* which he decided were brought about by bacteria; he named the organism *Bacterium moniliformans.* The original article has not been seen by the writer, but according to Sorauer (I, '08, p. 30) Guffroy has presented no proof for his conclusions.

Voglino (I, '05, pp. 43–44) described a bacterial disease of rice, present in Italy. The large size of the organism, 2.5–3.5 × 3.8–4.0 μ, should readily distinguish it from all other organisms attacking grasses.

Manns (I, '09) published an extensive account of a bacterial disease of oats, although Galloway and Southworth as early as 1890 and Russell in 1892 briefly described a bacterial disease of the same host. It may be noted that Manns' conclusions concerning the cause of the disease have been questioned, and more recently Miss Elliott (I, '20) has given a detailed account of a halo-blight of oats. Concerning the disease which Manns described, she says (p. 167); "His colored figures as well as most of his text indicate an entirely different disease, but his Plate

XIII shows that this halo-disease formed at least a part of the phenomenon under consideration." She states further (p. 168), that "Manns' descriptions of individual lesions are so meager and his descriptions of general signs so inclusive as to lead to grave doubt about his having worked with a single bacterial disease." This statement is somewhat different from the one made by Miss Elliott in a previous publication (I, '18). In this she says, "The characteristic halo lesions of this disease have been definitely observed in Wisconsin during each of the past five years and are similar to those figured by Manns (Pl. XIII) in his Ohio bulletin of 1909. The results of the writer's work on halo blight of oats agree with those reported by Jones, Johnson, and Reddy* ('17) in that typical halo lesions are readily produced by a white bacterial pathogen alone." In view of the facts that Manns had included a number of grasses as hosts and that Jones and his associates had accepted *Pseudomonas Avenae* as the pathogen I made the following statement in a preliminary note on the foxtail disease: "The effect on oats is not unlike the halo blight recently described by Miss Elliott and it is likely that the organism under discussion is the same as Manns' *Pseudomonas Avenae.* However the identity of the organism is still in doubt and the work is being continued."

The writer is now in a position to say that Manns' description of *P. Avenae* is quite at variance with the account of the foxtail organism which will be presented later. It must be borne in mind, however, that bacteriological methods and descriptions in 1909 were not as well developed as they have become in more recent years, and when, for example, endospores are recorded in 1909 it must not be assumed that this is an established fact; the same thing may be said for a large number of cultural reactions. However, while the foxtail organism may be present in Ohio, Manns' description as well as Miss Elliott's, even when interpreted very broadly, clearly indicates a difference in the organism. A comparison of group numbers, using the 1907 chart of the American Bacteriological Society, shows at a glance the marked difference between the foxtail organism and *P. Avenae.* Manns' group number (I, '09, p. 133) is 111.2223032, while the group number of the foxtail organism is 212.3333013. Among other differences it may be noted that the foxtail organism does not liquefy gelatin, produces no acid, even in the presence of car-

*These writers considered Manns' *Pseudomonas Avenae* as the causal organism.

bohydrates, and produces strong diastatic action, characters quite different from those described for *P. Avenae.*

Miss Elliott was kind enough to send the writer a culture of *Bacterium coronafaciens,* the causal agent of the halo-blight of oats, and comparison of this organism with the foxtail pathogen shows distinct differences. As Miss Elliott states, *B. coronafaciens* usually occurs in chains and produces a brownish color on nutrient agar; these characters serve to distinguish it immediately from the foxtail organism, which usually occurs singly or in pairs and produces no brown color on nutrient agar. There are also a number of other morphological and physiological differences which can be readily ascertained by comparing the description of the foxtail organism given below with the one given by Miss Elliott (I, '20). Before closing the reference to bacterial diseases of oats the writer wishes to call attention to a pathogen isolated by him from reddish spots of oats, an organism which when inoculated in pure culture, produced the same kind of spots (no halos are produced). This organism, a white, monotrichous rod, is a strong gelatin liquefier and does not attack foxtail. It apparently is not *Bacterium coronafaciens.*

A bacterial disease of western wheat-grass was described by O'Gara in 1915 (I, '15, '15[a]). The pathogen, *Aplanobacter Agropyri,* is yellow and non-motile and thus quite different from the foxtail organism.

In 1916 Jones, Johnson, and Reddy briefly described a bacterial blight of barley and certain other cereals. This was followed by a more complete description in 1917. In this, also, a yellow organism, *Bacterium translucens,* is involved. Besides barley, these writers have found that wheat, spelt, rye, timothy, and oats are attacked by similar bacterial diseases, but that the barley organism, *B. translucens,* does not attack the other grasses listed above. Attention has already been called to a bacterial disease of barley found in Germany by Gentner (I, '20). While proof has been given that *Bacillus cerealium* may produce disintegration of seed, it seems to me that more work would be necessary before any conclusion could be drawn regarding the production of brown spots on various organs of the barley plant. As indicated previously the organism differs greatly from the foxtail pathogen.

A new disease of wheat, now well known as black chaff, was first described by Smith in 1917. The pathogen is closely related to the one causing barley blight and has been named by Smith,

Jones, and Reddy (I, '19) *Bacterium translucens* var. *undulosum*. This organism goes as readily to barley as the barley organism itself, while the barley organism hardly attacks wheat. It also is a yellow pathogen.

Another bacterial disease of wheat was found in India by Hutchinson and first described in 1917. The descriptions indicate that the disease is quite similar to the one described by O'Gara (I, '15, '15[a]) on western wheat-grass. There is a marked distortion of the heads and the spikelets are covered by a yellow slime. The pathogenicity of the organism, *Pseudomonas Tritici* Hutch., has not as yet been definitely established. The symptoms, particularly the yellow slime, would indicate that it is quite different from the foxtail disease.

For convenience a third bacterial disease of wheat, the basal glumerot, may be considered next. It was discussed by Miss McCulloch in 1920, and the pathogen, *Bacterium atrofaciens*, described as a greenish pigment producer, is quite distinct from the foxtail organism.

In closing the historical resumé of bacterial diseases of grasses it should be noted that no attempt has been made to include all references, but rather those of significance in relation to the disease producer here investigated. The 14 grass genera listed by Smith (I, '20) have been included and besides these reference has been made to a bacterial disease of *Arrhenatherum* and of *Oryza*. In addition special attention may be called to the very brief notice of a bacterial disease of millet (listed under *Panicum* sp.[1] but perhaps intended to be *Chaetochloa* (*Setaria*) sp.) in a supplement bulletin of the Plant Disease Survey (I, '19, p. 157). The note reads: "Blight, apparently of bacterial cause, was noticed on three varieties of millet in Minnesota about August 17." This reference is of particular interest, since millet, *Chaetochloa italica* (*Setaria italica*), is closely related to foxtail, *Chaetochloa lutescens* (*Setaria glauca*) and in the seedling stage is as readily attacked by the foxtail organism as foxtail itself. Various other references have been found to bacterial diseases of grasses, including those which produced spots on rye and on corn, but these are so brief as to be of no special significance for present purposes.

[1]There are a number of different grasses going under the name of millet, but the names foxtail, millet, or Italian millet are applied to *Chaetochloa italica*, while proso or broom-corn millet are applied to *Panicum miliaceum*. The latter is but sparingly grown in this country. (See Hitchcock, U. S. Dept. Agr., Bur. Pl. Ind. Bul. 772. 1920.)

General Description of the Disease

The symptoms of the disease vary somewhat with host and considerably with conditions under which infection takes place. The pathogen grows best at relatively high temperatures, so that other conditions being favorable, at a temperature of around 90° F. the first signs of infection will occur in 24 to 48 hours. Under very moist conditions the spots appear water-soaked at first and as the atmosphere becomes dry they appear as light brown or grayish brown spots or streaks. On foxtail and on *Chaetochloa geniculata* these spots may be bordered by a distinct brown or reddish brown area (see pl. 23, fig. 1). Under favorable conditions of infection attacked areas often appear grayish green, withered, looking as if scalded, and surrounded by a somewhat indefinite yellowish halo. The spots on foxtail as they occur naturally in the field, in a dry atmosphere, are usually of two types, (1), small, reddish brown, round or oval-shaped areas enclosing lighter brown centers, and (2), light or dark brown or blackish streaks, usually starting at the tip of the leaf and often including a large part of leaf area. The spots may appear on any part above ground, including leaf-sheath as well as blade, rachis, and glume. Under natural conditions spots are more often to be observed on the leaf blades.

On oats the spots vary from light yellow, somewhat indefinite areas to grayish green, markedly withered areas. Often there are marked tinges of red[1] in attacked areas, particularly when a number of infections have coalesced. Attacks may occur on seedlings or on plants that are in head. Numerous artificial inoculations have been made on various varieties of oat seedlings, and under favorable conditions of infection not a single oat plant out of a large number inoculated would remain alive 4 days after inoculation (see pl. 26, fig. 1). It is of course evident that a seedling with a limited surface is much more readily killed than a larger plant.

Attacks on wheat, rye, barley, and corn do not vary considerably from that on oats. There is usually very little red discoloration, the spots vary in size and shape, and the predominant discolorations are grayish green, light yellow, or brownish,

[1]The writer is satisfied that various agents can cause reddening of oat leaves. Under certain conditions of light and temperature a mere sharp crease in an oat blade may produce a reddening in the part above the crease. It is well known that the production of anthocyans may take place at low temperatures and when the transfer of foods is cut off, resulting in an accumulation of sugars. Attacks by aphids often appear as yellow or red discolorations.

withered areas. On barley and on corn very light yellow or whitish streaks are not uncommon. Barley seedlings, as well as oats, succumb very readily (see pl. 26, fig. 2).

On millet the attack is usually in the form of grayish green, withered areas, occasionally with brown tinges. On various varieties of sorghum the spots are often grayish green bordered by a red band or merely a small red, irregular spot; reddening, it may be noted, being a much more general character of various sorghum diseases.

There are two diseases of foxtail which are of common occurrence in Arkansas as well as in other states, notably Delaware, Missouri, New York, and Pennsylvania, which may be mistaken for the bacterial disease. One of these is caused by *Cercospora Setariae* Atk. and the other is a *Piricularia* spot. The *Cercospora* attack is usually in the form of elongated dark brown streaks which usually exhibit the spore groups and thus are easily identified, while the *Piricularia* spot is usually roundish, light brown in color with a darker brown edge. These *Piricularia* spots may readily be confused with the bacterial spot because of their close resemblance and because the spores are often difficult to find. Microscopic examination will, however, usually bring out the difference. The bacterial spots are full of bacteria which in a water mount with slight pressure will stream out of the tissues in the form of dense, grayish white clouds. They may also be detected within the cells. Rarely are bacterial spots contaminated by fungi.

INTERNAL APPEARANCE AND PATHS OF INFECTION

Sections of diseased leaves show the bacteria present in great numbers within the cells and in the intercellular spaces (see pl. 27, fig. 1). The parenchyma is apparently the only part attacked, since no bacteria have been observed within the bundles. The attacked cells lose their turgidity, suffer partial or complete collapse, are more or less disintegrated, and variously discolored. Externally the collapse of cells is indicated by a shrinking and withering and finally by splits or breaks in the diseased parts. Ordinarily there is no oozing outward of bacteria, but under very moist conditions and where rifts have occurred in the tissues the bacteria are present in great numbers in the drops hanging to the rifts. This undoubtedly leads to the dissemination of the pathogen by rain and by winds. (Spreading by insects is also to be expected.)

Artificial inoculations clearly indicate that the pathogen enters by means of the natural openings, the stomata and the water-pores. Material fixed in chrom-acetic acid, imbedded in paraffin, sectioned, and stained with carbol-fuchsin has shown substomatal cavities full of bacteria, which in later stages of infection are surrounded by cells and intercellular spaces which are also full of bacteria (pl. 27, fig. 1). Regarding infection through water-pores it was noted that under certain conditions in the greenhouse, which included an extreme dryness of the atmosphere, resulting in an incipient wilting accompanied by a closing of the stomata, the few infections obtained occurred at the tips of the leaves, indicating entrance by means of the apical hydathode. This is of interest since it indicates that under natural conditions when stomata are closed, as at night, infection may take place through the water-pores.

HOSTS AND EXTENT OF INJURY

Thus far, foxtail is the only grass that has been found infected under natural conditions. This statement may not be of much significance, as the time spent in looking for the disease on other hosts was, by force of circumstance, very limited. The ease with which artificial infections are obtained on a number of wild and cultivated grasses indicates that the pathogen may be looked for on all the common cereals, particularly in the southern states. The following species representing 5 different tribes are susceptible, as proved by artificial inoculations: *Avena sativa, Chaetochloa geniculata, Chaetochloa italica, Chaetochloa lutescens, Holcus Sorghum* (*Andropogon Sorghum*), *Holcus Sorghum sudanensis, Hordeum vulgare, Secale cereale, Triticum sativum,* and *Zea Mays.*

A sufficient number of artificial inoculations and of reisolations have not been conducted with red top (*Agrostis palustris*) and with goose-grass (*Eleusine indica*) to warrant any definite statement, but preliminary tests would seem to show that these also are susceptible.

Artificial inoculations have given negative results with the following: *Poa pratensis, Syntherisma sanguinalis, Chaetochloa viridis, Festuca elatior, Oryza sativa,* and *Phleum pratense.*

The disease on foxtail has now been observed in northwest Arkansas for four successive seasons. Specimens have also been obtained from other portions of the state, indicating that the

disease is widespread throughout Arkansas. No effort has so far been made to locate the disease in other states, so that little is known concerning its distribution. It should be looked for particularly in the warmer sections of the country, since physiological studies indicate that the organism grows best at relatively high temperatures. Field infections on foxtail are at times severe, especially during warm, moist weather. Under such conditions it is not uncommon to find individual plants with almost every leaf-blade killed and with only the rachis and head showing a normal, green color. Lower leaves in particular are to be found diseased (indicating infections by spattering of rain drops). However, considering the extent of injury as observed over a period of several years there is no reason for viewing this disease with alarm. On one particular field where it has been under close observation for more than three years, the disease has occurred on 4 successive crops of foxtail without any apparent diminution in the number of volunteer plants that sprang up. Every year this field had been plowed, cultivated, and used for growing tomatoes and other crops, and by late summer, unless considerable hoeing were done, foxtail would "take" the field. In this connection it is worth while calling attention to the distribution and growing season of the host. Yellow foxtail is common in cultivated soil in the eastern United States (it is also very common in the Mississippi region) according to Hitchcock (U. S. Dept. Agr. Bull. 772, p. 243) and is "often sufficiently abundant to furnish considerable forage." It usually does not appear before midsummer and comes into full development by late August or early September. Thus we see a high, midsummer temperature favoring host development as well as that of parasite.

SUSCEPTIBLE VARIETIES OF COMMON CEREALS

The following varieties of common cereals[1] have been found susceptible in artificial infection experiments by methods which will be explained below. For each variety listed one or more pots each containing numerous plants were inoculated. When infections were few or uncertain other pots of the same variety were tried. Uninoculated plants served as checks. Because of rapidity of growth as well as ease of handling, seedlings 6–12

[1]The writer wishes to express his indebtedness to the Agricultural Experiment Station of the University of Arkansas and in particular to Professor W. H. Sachs for supplying most of the seed of the varieties listed. The varieties are for a large part those that are adaptable to Arkansas conditions.

inches high were largely used. (Numerous inoculation experiments show that maturer plants may be also infected.)

Wheat: Alabama Blue Stem, Coker's Blue Stem, Black Hulled, Fulcaster, Fultz, Georgia Red, Gladden, Golden Chaff, Gypsy, Harvester King, Hastings, Jones' Climax, Leap's Prolific, Lebanon, Marvelous, Medium Mediterranean, Michigan Wonder, Poole, Portage, Purple Straw, Red May, Red Wonder, Stover's Miracle, Turkey, Nebraska No. 6, and Turkish Amber.

No infections were obtained on Bartt, Beechwood Hybrid, Longberry, and Red Rock, but it should be noted that experiments would have to be repeated with these varieties before any deductions were made, since the temperature prevailing in the greenhouse at the time inoculations were conducted was considerably below the optimum for infection.

Oats: Appler, Ferguson, Virginia Turf, Wilson, Winter Gray, and Winter Turf.

Rye: Abruzzi, Rosen, Station, Texas Winter, and Winter Minnesota. Infections on Ivanhoff No. 34 not obtained.

Barley: Mancheuri, Oderbrucker, and Wisconsin Pedigree No. 6.

Corn: Arlington Prolific, Biggs' Seven Ear, Calhoun Red Cob, Chisholm, Coker's Ellis, Coker's Marlboro, Coker's Prolific, Coker's Williamson, Eureka, Experiment Station Yellow, Hickory King, Jarvis' Improved, Laguna Mexican June, McFarland, Mosby's Prolific, Sentell's White Dent, Singleton's Strawberry, Stewart's Yellow Dent, Surecropper, Thibault's Mexican June, Weekby's Improved, Whattey's Prolific, and White Wonder.

Varieties of corn upon which no infections were obtained are Brazos White Corn, Coker's Garric, Ewing's Mosby, Paymaster, Silvermine, and Southern Beauty. Time has not permitted any adequate study which would definitely show whether or not these are resistant.

Sorghum: Black Amber, Darco Non-saccharine, Honey, Red Amber, and Sugar Drip.

The following sorghum varieties yielded no infections: Broom Corn, Silvertop, Shrock Kafir, Sumac, and Sunrise Kafir. Here also conditions for infection were not the best.

SUMMARY OF THE WORK ON VARIETAL SUSCEPTIBILITY

The following points may be emphasized: first, that a comparatively large number of varieties of different cereals are susceptible; second, that the degree of susceptibility has not been

clearly worked out; third, that certain cereals, like oats, barley, and rye, seem to show greater susceptibility, and conversely, that others, like corn and sorghum, are not as susceptible. The infection experiments here reported were done entirely in the greenhouse, and at times factors influencing infection, such as temperature, were difficult to control, so that conditions for infection were not uniform. The degree of susceptibility was measured by the number and size of the spots produced.

Isolation and Inoculation Experiments

The organism was not difficult to isolate, and when once its identity was established and the peculiar behavior of producing a colorless halo surrounded by a white precipitate on certain culture media was recognized, it was easy to detect it on a poured plate, even in the presence of various other *Schizomycetes*. During the 4 years in which this disease has been studied, the pathogen has been isolated many times from natural infections and from artificial infections on various hosts, using the ordinary method of surface sterilization with 1-1000 mercuric chloride solutions for about 2 minutes, washing in several changes of sterile water, macerating aseptically either in sterile water or in beef bouillon, diluting variously by successive transfers into sterile water blanks, and finally by making poured plates containing small amounts of the dilution culture in nutrient agar. When proper precautions are taken, such as a thorough cleansing of hands, of clothing, and of the chamber in which the work is conducted it is common to get a series of plates in which the pathogen only is present.

Since the organism is very sensitive to alcohol, as will be shown later, attempts at surface sterilization with alcohol-mercuric chloride solution are apt to yield no colonies, although the writer has been able to obtain isolations in this way by a very rapid transfer of the diseased material from the alcoholic solution to the straight mercuric chloride solution.

In inoculation experiments the organism was at first smeared on the leaves by means of a sterilized platinum loop and in all later work was applied as a spray. The first method has the advantage of enabling one to locate definitely the point of inoculation in relation to the point of infection, but it is rather tedious when any number of inoculations are to be attempted. Either agar or broth cultures (the age of the cultures, within limits,

not being very important for this organism) were diluted with sterile water before the spray was applied. (In smear inoculations the plants were first sprayed with sterile water.) The spray was obtained by using atomizers or blowers commonly used by artists in "fixing" drawings. These work very rapidly and effectively, producing a fine mist with little effort.[1] The organism having been applied, the plants were then covered with bell jars to prevent drying out. The bell jars were ensconced with paper in order to cut down direct sunlight. The jars were left over the plants for 1 or 2 days, depending on how high the temperature happened to be. It may be worth recording that the difficulty experienced in obtaining infections in the experimental greenhouses of the Missouri Botanical Garden, although no such difficulty had been experienced at Fayetteville, Ark., was overcome by proper aëration. The greenhouse benches at the Garden are of concrete. When inoculated pots of plants were placed on soil which filled these benches, and the bell jars which covered the plants made close contact with the soil, infections were rare, no matter what the temperature and humidity happened to be. But by raising the jars from the soil on small blocks of wood the plants were still kept moist and at the same time had access to air. In this way infections were readily obtainable, other conditions being proper.

RELATION OF TEMPERATURE TO INFECTION

It has already been indicated that temperature plays an important role in infection. The reproduction of thermographs of artificial inoculations on Winter Turf oats carried out as previously described, illustrates this point (fig. 1).

The pot of plants subjected to the inoculation temperatures shown in the lower graph developed no infections, while that of the upper developed quite a few. It will be noted that where no infections were obtained the temperature over a large part of the 48-hour period was below 70° F. (21° C.), and particularly during the first 24-hour period the temperature was below 70° F. for 18 hours. The atmosphere was saturated and the leaves were covered with water films in both cases. It was found difficult to keep the incubator at an even temperature, so that no data are available which will show the relationship of infection to a definite temperature. Nevertheless, the graphs give some in-

[1]Dr. J. A. Elliott first suggested these blowers to me. They are cheap, easily sterilized, take up little space and do not plug readily.

dication on this point and when one considers the difference between night and day temperatures often encountered in the field the data shown are perhaps more indicative of infection to be expected under natural conditions than any experiment would show in which the temperature is kept at one level during the entire incubation period. Roughly then, it may be said that tem-

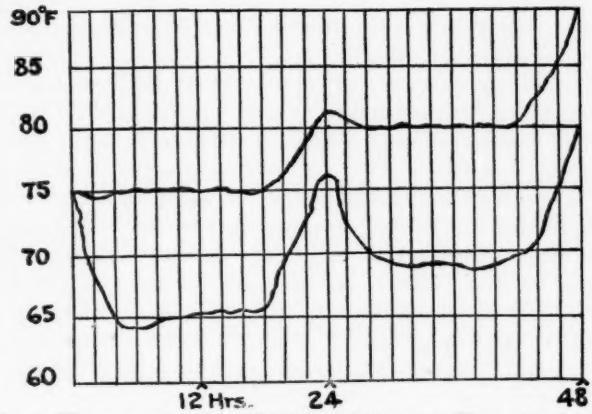

Fig. 1. Thermographs of artificial inoculations on Winter Turf oats.

peratures, during the infection and incubation periods, below 70° F. prevent infection, while temperatures above 75° F. are conducive to infection. It may be added that numerous artificial infection experiments show that heaviest infections occur at temperatures between 85 and 95° F. (29 and 35° C.). This is to be expected since growth in artificial culture media is best at these temperatures, as will be shown later.

THE ORGANISM

Morphology.—The organism is an actively motile, medium-sized rod. Whether obtained directly from the host tissue or whether grown on artificial media of various kinds no marked differences in morphology have been observed. It is rather slender (pl. 27, fig. 2) with rounded ends, and measures 0.5–0.8× 1.5–2.0 μ, 0.6×1.8 μ being very common measurements. Very rarely is it observed other than singly or in pairs, although occasionally a short chain of 4 or 6 organisms is to be seen. Its motion, which is very rapid in warm water, is due to a single, polar flagellum which varies somewhat in length but is commonly 3 to 5

times the length of the organism. Van Ermengem's stain brings out the flagella, but unfortunately it does not stain the body of the organism properly (pl. 27, fig. 3). Pitfield's stain as well as Shunk's (II, '20) modification of Loeffler's stain, has also shown but one polar flagellum. The latter stain, in particular, after the mordant has been seasoned for several weeks, forms an excellent stain for this organism, the disadvantage being that the blue color imparted by the methylene blue is difficult to photograph by ordinary methods.

The organism possesses capsules, as definitely shown by using Huntoon's (II, '17) method, "nutrose" being used as the diluent and Ziehl's carbol fuchsin added in addition to the stain recommended by Huntoon. This method, in contrast to Ribbert's or Welch's, is much more satisfactory in bringing out the capsule on this organism. Young and old cultures of the organism on nutrient agar slants and in bouillon were used, and capsules were observed in every case. No zoogloea or pseudozoogloea were formed, although in old cultures of beef-bouillon and particularly in bouillon containing various sugars, a dense precipitate settled, which on agitation rose in whorls and was broken up with difficulty.

The organism has not been found to produce spores. Cultures at various ages and on various media were examined. Anjesky's spore-staining method, recommended by Giltner ('Laboratory Manual,' 1921 edition) was used. It is not acid fast. A bouillon culture 3 days old stained with Ziehl's carbol-fuchsin and then treated with 20 per cent sulphuric acid for a moment, showed no color. When counterstained with aqueous methylene blue the organism appeared deep blue with no trace of red. It was Gram negative. A 24-hour culture treated with the Gram stain was colorless and became brown when counterstained with Bismarck brown. A check test with the same Gram stain on *Staphlylococcus albus* showed this organism definitely stained. Ordinary stains, like gentian violet, carbol fuchsin, methylene blue, and Bismarck brown were taken up readily. No irregular or involution forms have been seen.

CULTURAL CHARACTERS IN RELATION TO ACIDITY AND ALKALINITY

Methods.—In making up culture media the recent recommendations of the committee (Conn, II, '20) representing the American Bacteriological Society, as far as they go, were followed, with modifications as will be noted, and in addition other methods

and media were used which seemed worthy of trial. The aim has been to utilize the current methods of plant pathologists and at the same time take cognizance of technique employed by bacteriologists in general and by the medical men in particular.

The necessity of utilizing methods which are not commonly used by plant pathologists became evident early in the work, and having in mind the busy life of the ordinary pathologist, it will be necessary to indicate the reason for the adoption of any new scheme.

Using nutrient agar testing +10 or +15 on Fuller's scale (10 or 15 parts per 1000) the writer found that from diseased foxtail material he would at times obtain bacterial colonies which produced a very conspicuous reaction, a colorless zone followed by a marked whitish area (pl. 28, fig. 1), and then perhaps at the same time, on other plates, or at other times, using the same medium, colonies were obtained without any such features. Morphologically, the colonies as well as the individual bacteria appeared exactly alike, and inoculation experiments showed that both were pathogens producing the same kind of spot. Two explanations for this variation seemed plausible, first, that there existed 2 strains of the same species, one producing a precipitate and the other not, and second, that the conditions in the nutrient media were different. It was soon found that a colony that produced a white precipitate may, on transferring to another plate or tube, show no such character. It appeared therefore that there must be some slight difference in culture media even though it was made up at the same time and received the same treatment throughout.

In an effort to solve this, and mindful of the extensive data that were accumulating on hydrogen-ion concentration in relation to various phases of bacterial activity, the writer instead of titrating by the Fuller scheme, using phenolphthalein as the indicator, used the indicators recommended by Clark (II, '20) in which the color change with reference to acidity or alkalinity had been definitely worked out in terms of hydrogen-ion concentration. The color obtained by adding a few drops of indicator, like brom thymol blue, to a tube of melted, nutrient agar was then compared with the color chart given by Clark (following p. 40), and the color in the series for brom thymol blue matching best with the tube was taken as the hydrogen-ion concentration in terms of P_H. This of course is a rough way of measuring true

acidity or alkalinity but its superiority over Fuller's method soon became evident. Nutrient agar which gave a yellow or yellowish green color with brom thymol blue (those covering the reaction range of P_H 6.0–P_H 6.8 and therefore acid) when used as a substrate always showed colonies producing a colorless region followed by a dense precipitate, while agar which gave a blue color with brom thymol blue (those having a reaction of P_H 7 or over, hence neutral or alkaline) when used as the substrate showed colonies which never produced any precipitate.

The presence or absence of a colorless zone plus precipitate could then be controlled at will, depending on whether the medium was rendered slightly acid or slightly alkaline as measured by the hydrogen-ion concentration. Here then is a method of measuring acidity and alkalinity which is just as simple as Fuller's and much more accurate. By using this method the writer was able to obtain a character which immediately distinguished this bacterium from other similar-appearing organisms; in other words, using a medium of P_H 6.6, no matter what other white colonies might develop on a given plate, this organism could readily be recognized. (This power of producing a colorless zone and a white precipitate was an excellent character as a quick test for differential purposes).[1] The reason why Fuller's scale did not bring out this feature is apparent. A medium which tests +10 on Fuller's scale, and thus supposedly acid, is often, as will be shown later, really neutral or slightly alkaline. Besides this, it has been found that a medium which is slightly acid when it is prepared will change its reaction upon sterilization or upon standing. In particular the writer has found that nutrient agar kept in glass tubes of various "makes" will in time become alkaline.[2] This is especially true of much of the soft glassware obtained during the war, so that media that tested P_H 6 when made would, after two weeks, test P_H 7, and moreover, the same degree

[1] Miss Bryan (II, '21) has recently described a bacterial bud rot of cannas in which she shows the pathogen producing a colorless zone surrounded by a white precipitate on whey agar. Because of this similarity with the foxtail organism it was thought desirable to compare *Bacterium Cannae* with the foxtail pathogen. Miss Bryan very kindly furnished the writer with cultures of her organism. Comparison between the two show numerous differences, morphological and physiological, although as far as producing a precipitate is concerned they behave alike. The interesting thing about *B. Cannae* is that it produces a colorless area surrounded by a white precipitate not only on whey agar but also on ordinary nutrient agar possessing a slight acidity, as P 6.6. Furthermore, the production of this character can be controlled in the same manner as in the foxtail organism.

[2] Esty and Catheart (II, '21) have recently given some interesting data on the effect of different glassware on various solutions.

of change did not occur in different batches of tubes. New tubes in particular, even after hardening in several dippings of sulphuric acid-potassium dichromate cleanser and after several sterilizations in the autoclave, persisted in giving off considerable alkali. Hence in any series of 10 tubes, some made by one concern and some by another, or some old and others new, it was possible to obtain various acidities and alkalinities using the same medium. This in all probability is the reason for obtaining a series of poured plates with the same organism, some of the plates showing colonies with characteristic precipitate and others lacking in this feature. For the effect of autoclaving on the reaction of the medium see articles by Clark (II, '15, p. 130), Anthony and Ekroth (II, '16), Norton (II, '19) and Davis (II, '20). Besides glassware, other factors may change the reaction of a medium. Grace and Highberger (II, '20) noted changes in acidity, particularly in uninoculated glucose broth, and offered no explanation for the change. Foster and Randall (II, '21) found an increase in acidity of broth which they concluded was due not to a taking up of carbon dioxide, but to an opening up of the COHN groups as a result of the hydrolysis of the protein constituents.

Here it may be pointed out that not only has titration as measured by the hydrogen-ion concentration given more accurate results but the use of this method has stimulated careful analyses of physiological processes as related to active acidity or alkalinity with the discovery of numerous interesting and often valuable relationships, particularly with reference to human pathogens. A few instances will be cited. Clark and Lubs (II, '15) differentiated between organisms of the colon-aerogenes group by the use of indicators whose color changes were noted in terms of hydrogen-ion concentration. Ayers (II, '16) found that streptococci reached a more or less definite hydrogen-ion concentration in culture media, a fact which, he decided, helped in the classification of these bacteria. Later, in 1918, Ayers, Johnson, and Davis found that a non-pathogenic strain of *Streptococcus* reached a higher hydrogen-ion concentration than the pathogenic strains. They concluded that the difference in hydrogen-ion concentration may be used as one means of distinguishing non-pathogenic from pathogenic streptococci. Finally, Avery and Cullen (II, '19) found that they were able to distinguish human from bovine strains of *Streptococcus* because of the fact that human strains in nutrient broth develop a lower hydrogen-ion concentration.

They were thus enabled to differentiate between those organisms that may be pathogenic to man and those that are not.

Fennell and Fisher (II, '19), working with cultures of pneumococcus and meningococcus, were often unable to obtain suitable growth although the media utilized had apparently the same composition, was made in the same way, and the titration by the Fuller method was always adjusted to the same point, +0.2 (0.2 per cent). Using the hydrogen-ion concentration in their titration they obtained profuse and consistent growth. They found that pneumococcus has a very narrow range for optimum reaction, P_H 7.8 to P_H 8.0, which they considered as equal to +3.0 to +3.5 (3–3.5 per cent), "a total acidity far beyond anything previously suggested for the growth of pneumococcus." Norton (II, '19) obtained similar results.

Not only has the hydrogen-ion concentration been used successfully for distinguishing between closely related organisms, but its use in the study of various phases in metabolism has yielded valuable results. Its relationship to the growth of pneumococcus and meningococcus has just been pointed out. In addition it may be noted that Cole and Lloyd (II, '17) showed that among other factors, a suitable hydrogen-ion concentration is important for the cultivation of gonococcus. There are now numerous records like these which show the relationship of growth and development of many different micro-organisms to hydrogen-ion concentration. Concerning its relationship to definite physiological reactions there are also numerous records; for example, Bronfenbrenner and Schlesinger (II, '19) pointed out that the amount of acid and gas produced by bacteria depended on various factors, including the amount of carbohydrate, the amount of peptone, the amount of buffer, and on the hydrogen-ion concentration; Bigelow and Esty (II, '20) showed that a slight change in the hydrogen-ion concentration greatly affected the thermal death point, and Chambers (II, '20) gave accurate counts of the number of bacteria developed at different levels of hydrogen-ion concentration. Studies on non-pathogenic bacteria and fungi, as well as on higher plants, have shown similar relationships to hydrogen-ion concentration. The work of Allen (II, '19) on *Azotobacter,* Gillespie and Hurst (II, '18) on *Actinomyces scabies,* Rose (II, '19) on *Nummularia discreta,* and Duggar (II, '20) on nutrient solutions for higher plants, to mention but a few, has

shown the importance of hydrogen-ion concentration in the metabolism of numerous and diverse organisms.

Clark in 1915 summed up the matter as follows (p. 109): "The rate of enzyme action, the stability of colloidal structures upon which cellular life depends, the solubility of many physiologically important compounds, as well as the structure and composition of media and the color of the indicators used in the adjustment of their reactions all are dependent in greater or less degree upon hydrogen-ion concentration."

While it is absolutely essential that plant pathologists recognize the importance of the determination of hydrogen-ion concentration, yet it should be recognized that other factors are equally as important and when it is desired to know the buffer strength of a medium it even becomes necessary to use total acidity titration. That there is danger of attributing too much to hydrogen-ions has been pointed out in recent articles by Brown (II, '21[a]), Jones (II, '20), and Traube (II, '21).

In spite of the very considerable amount of data that has accumulated on this subject, many plant pathologists, as has already been stated, have paid little attention to it. With very few exceptions, Wolf and his associates in particular (II, '21), those who have dealt with bacterial plant pathogens have more or less completely ignored all the recent advances in this phase of bacteriology. Having presented data showing the necessity for determining the hydrogen-ion concentration of bacteriological media the writer will attempt to outline very briefly the principles involved and the methods used. This has already been done in a number of articles and books; the aim in presenting the matter here is to make available to plant pathologists the information at hand and to call attention to the more important references.

PRINCIPLES INVOLVED IN THE DETERMINATION OF ACID AND ALKALI

To define the terms acid and alkali in relation to the color reaction of some organic indicator is not satisfactory. Thus, to say that a solution is acid because it shows no color in the presence of phenolphthalein does not give much information, since by using another indicator, such as Congo red, the same solution may be said to be alkaline (see Washburn, II, '10). The satisfactory basis for a definition of acidity and alkalinity is furnished

by the ionic theory of Arrhenius (II, '13)[1]. According to this theory when a strong electrolyte dissolves in water in a dilute solution it is highly dissociated into two ionic groups. Hydrochloric acid, for example, breaks up into positive + H ions and negative — Cl ions, and sodium hydroxide breaks up into + Na ions and — OH ions. When a solution of hydrochloric acid is mixed with a solution of sodium hydroxide the hydrogen ions combine with hydroxyl ions to form water and in this union neutralization takes place. Pure water itself dissociates into + H and — OH ions, and the dissociation yields equal amounts of + H and — OH ions. A neutral solution may therefore be defined as "one in which the concentration of hydroxyl ions is equal to the concentration of the hydrogen ions. From this it follows that an acid solution is one in which the concentration of hydrogen ions exceeds that of OH ions, while an alkaline solution is one in which the concentration of hydroxyl ions exceeds that of hydrogen ions." Strong acids and alkalis like hydrochloric acid and sodium hydroxide are almost completely dissociated at certain concentrations, while weak acids like acetic acid and weak alkalis like ammonium hydroxide are only slightly dissociated.

The extent to which substances are dissociated into their ions may be determined by the hydrogen electrode and by other methods, and this determination, in which the hydrogen- and hydroxyl-ion concentration is definitely determined, becomes a measure of the acidity or alkalinity of the solution.

Normal solutions of hydrochloric acid and of acetic acid will neutralize the same amount of sodium hydroxide, but since hydrochloric acid is a strong acid it is almost completely dissociated, while the weak acetic acid is but weakly dissociated. The result is that normal hydrochloric acid, according to Michaelis (II, '14, p. 23), contains about 0.8 gram of hydrogen per liter, while normal acetic acid contains only 0.0043 gram of hydrogen per liter. The difference between the 2 acids may be noted in table I, taken from Michaelis (II, '14, p. 23).

As has already been indicated, water itself is an electrolyte and dissociates into + H ions and — OH ions. Sörensen determined that pure water at 22° C. contains a concentration of 1/10,000,000 of either ion, that is, that it contains 0.0000001 gram of H ions and the same amount of OH ions per liter. In other words

[1]This author, in a comparatively simple account, has summarized in English, the theories of solutions. The book is easy to read and is not burdened with mathematical formulae.

pure water is 0.0000001 normal acid and at the same time 0.0000001 normal alkali. Such fractions as 1/10,000,000 (0.0000001) being unwieldy, the logarithmic equivalents are used; thus 1/10,000,000 may be written 10^{-7}, and the hydrogen-ion concentration of theoretically pure water may then be written 10^{-7}, or expressed in the form of a negative logarithm as Sörensen does, P_H7, which denotes the pressure of hydrogen ions (written variously pH, P_H, p_H^+, the last being Sörensen's original method [II, '09, p. 4]) or, in other words, the hydrogen-ion concentration.

TABLE I
H-ION CONCENTRATION OF STANDARD SOLUTIONS OF ACIDS

HCl at 18° C.			Acetic Acid at 18° C.		
Concentration	H-ion concentration	P_H	Concentration	H-ion concentration	P_H
Normal (1.0)	0.8	0.10	Normal (1.0)	4.3×10^{-3}	2.366
0.1 N	0.084	1.071	0.1 N	1.36×10^{-3}	2.866
0.01 N	0.0095	2.022	0.01 N	4.3×10^{-4}	3.366
0.001 N	9.7×10^{-4}	3.013	0.001N	1.36×10^{-4}	3.866
0.0001N	9.8×10^{-5}	4.009			

At a given temperature the product of the concentration of H and OH ions is constant and this value is called the "dissociation constant." For pure water at 22° C. this is equal to 10^{-14}, since, as has already been stated, the concentration of H ions and of OH ions is each equal to 10^{-7}. As the dissociation constant at a given temperature is always the same, the concentration of either +H or —OH can be expressed in terms of the other. Thus 1/100 N hydrochloric acid which has an hydrogen-ion concentration of 10^{-2} and an hydroxyl-ion concentration of 10^{-12} is said to have a value of P_H 2. Similarly, a 1/100 N sodium hydroxide solution which has an hydroxyl-ion concentration of 10^{-2} and an hydrogen-ion concentration of 10^{-12} has a value of P_H 12. All acids having a greater hydrogen-ion concentration than pure water have a value less than P_H 7 (the smaller the logarithmic exponent of a number less than 1 the greater the number); conversely all alkaline solutions having a lower hydrogen-ion concentration than pure water have a value greater than P_H 7. Table II adopted with modifications from Medalia (II, '20, p. 433) may help in an understanding of the matter.

Good accounts of the principles involved in hydrogen-ion concentration may be had in the following: Sörensen (II, '09), Wash-

burn (II, '10), Michaelis (II, '14), Clark (II, '15, '20) and Committee on Descriptive Chart for 1918 (II, Conn, '19).

TABLE II

RELATION OF P_H VALUES TO STRENGTH OF SOLUTION

Strength of solution	Grams of hydrogen per liter	Expressed logarithmically	P_H value	
Normal HCl	1.0	10^{0}	0.0	Acid
1/10 N HCl	0.1	10^{-1}	1.0	Acid
1/100 N HCl	0.01	10^{-2}	2.0	Acid
1/1000 N HCl	0.001	10^{-3}	3.0	Acid
1/10000 N HCl	0.0001	10^{-4}	4.0	Acid
1/100000 N HCl	0.00001	10^{-5}	5.0	Acid
1/1000000 N HCl	0.000001	10^{-6}	6.0	Acid
Pure water	0.0000001	10^{-7}	7.0	Neutrality
1/1000000 N NaOH	0.00000001	10^{-8}	8.0	Alkaline
1/100000 N NaOH	0.000000001	10^{-9}	9.0	Alkaline
1/10000 N NaOH	0.0000000001	10^{-10}	10.0	Alkaline
1/1000 N NaOH	0.00000000001	10^{-11}	11.0	Alkaline
1/100 N NaOH	0.000000000001	10^{-12}	12.0	Alkaline
1/10 N NaOH	0.0000000000001	10^{-13}	13.0	Alkaline
Normal NaOH	0.00000000000001	10^{-14}	14.0	Alkaline

METHODS OF MEASURING HYDROGEN-ION CONCENTRATION

The hydrogen-ion concentration, that is, the true acidity of a solution, may be measured in various ways. The two common methods used are, first, by measuring the electromotive force of a solution, and second, by the use of certain indicators whose color changes in relation to various levels of hydrogen-ion concentration have been definitely determined by the first method. The first or electrometric method, with good equipment, is more accurate, while the second or colorimetric method, while not as accurate, is much more available, since the cost is much less, and its operation very much more simple. (The writer having seen both in operation is satisfied that for ordinary work, and even for special research, the first is not essential for a bacteriologist.) For references to methods and apparatus necessary for electrometric measurements see Clark (II, '20) and Leeds and Northrup Company, Catalogue No. 75 (II, '20).

Indicators developed by Clark and Lubs ('17) for colorimetric work are especially well fitted for bacteriological media since they are as a whole brilliantly colored, their color changes at different levels are marked, and they are readily procurable at reasonable rates. Their color changes with reference to hydrogen-ion con-

centration have been carefully ascertained by Clark and Lubs and the values given by these authors have been checked up by numerous investigators. Explanation of the colorimetric method may be had in Friedenthal (II, '04), Salm (II, '04), Sörensen (II, '09), Clark (II, '15, '20) and Committee on Descriptive Chart for 1918 (Conn, II, '19).

STANDARDS FOR COMPARISON

Having added a few drops of an indicator, like brom thymol blue, to a tube of nutrient broth, how is the P_H value to be determined? The writer has already indicated one method, namely, comparing the tube with the color chart given by Clark (II, '20). This method for general laboratory work was found very satisfactory; the error was small, particularly if the solution was not highly colored; it was comparatively easy to manipulate and could be used readily in the class room; and best of all, it was very simple and comparatively inexpensive. The errors involved in this method are: first, it is difficult to get 2 charts which exactly agree in the shades of color for any one indicator, second, any color in the solution to be tested interferes with the color of the indicator. Nevertheless, in the absence of definitely known standards, this method is to be preferred, in the opinion of the writer, to the method advocated by the Committee on the Descriptive Chart (Conn, II, '20). This committee recognized the value of the Clark and Lubs indicators but instead of using anything for comparison, they simply recommend the following: "Bring the media to such an acidity as to turn this indicator (brom thymol blue) a distinct grass-green (neither yellow green nor blue green)."

It is evident from what has been said that it is desirable to have some standards whose hydrogen-ion concentrations have been definitely ascertained. Standards such as those recommended by Clark and Lubs contain certain salts, such as borates, phosphates, phthalates, which in the presence of certain indicators present definite colors. (The hydrogen-ion concentration of these standards having been previously determined by Clark and Lubs and by others using the electrometric method, it thus becomes possible in performing a titration to obtain a series of solutions of known hydrogen-ion concentrations which possess definite colors at certain levels of hydrogen-ion concentration.) The standards largely developed by Sörensen, recommended by

Clark and Lubs, and in common use are difficult and laborious to make, but for careful work, are apparently indispensable. Using these standards and a comparator block, it is a simple affair to compare a tube of media containing a certain indicator with another tube containing the standard solution plus the same indicator. This method, slightly modified by placing a tube of clear water before the solution to be tested and a tube of the unknown solution without indicator before the standard, as recommended by various investigators, has been extensively used by the writer in this investigation. The procedure is fully described by Clark (II, '20).

Various devices designed to simplify the procedure in making standards have appeared; most of these consist in reducing the number of standard solutions, which in some instances, also reduce the range of P_H values (see McIlvaine, II, '21). One of the simplest of these, judging from the description, is the one advocated by Acree and his associates (II, '21). The writer has not been able to use this. Other attempts to reduce the labor involved in making standards consist in varying the number of drops of indicator in a series of tubes containing simply a few cubic centimeters of acid and of alkali. This was first recommended by Barnett and Chapman (II, '18) and later amplified by Medalia (II, '20) and by Gillespie (II, '20). Bunker and Schuber (II, '22) claim good results by this method, and it is the one recommended by the American Public Health Association in the "Standard Methods for the Bacteriological Examination of Milk," 1921.[1]

BUFFERS

Standard solutions used for comparison with unknowns are often called "buffers." What is a "buffer"? Any solution which resists change in hydrogen-ion concentration upon the addition of acid or alkali is called a "buffer." It is of course desirable that standard solutions once made up should retain their calculated hydrogen-ion concentration as long as possible, and certain salts in particular are chosen for standards because of their marked resistance to change in hydrogen-ion concentration, even after standing for several months. Not only do a large number of organic and inorganic salts act as buffers but many other sub-

[1]Ready-prepared standard solutions are advertised by La Motte Chemical Products Company, 13 W. Saratoga St., Baltimore, and by Graham Chemical Company, 100 Rockingham Street, Rochester, N. Y. The latter concern sells the product developed by Acree and his associates ('21).

stances, such as peptone, beef extract, blood, etc., act in a similar manner. A good account of buffer action appears in Clark's work (II, '15, p. 116).

COMPARISON BETWEEN FULLER'S SCALE AND HYDROGEN-ION CONCENTRATION

Having briefly and perhaps inadequately described what is meant by hydrogen-ion concentration and how to measure it, the next question worthy of attention is, how does the older titration method, such as Fuller's (II, '95), compare with the determination of hydrogen-ion concentration. There are two main reasons why the two methods yield different results; first, the difference in degree of dissociation of different electrolytes; and, second, the buffer action of various substances. As previously described, when equal amounts of normal solutions of hydrochloric and acetic acids are titrated the same amount of alkali is utilized, although the two acids have entirely different P_H values, since one dissociates very strongly and the other but weakly; that is, titrating with sodium hydroxide, in the case of weak acids, gives no indication as to the actual acidity or the hydrogen-ion concentration. When a nutrient medium is neutralized with strong alkali the figure obtained gives no indication of the actual acidity present in the medium but gives an expression of the total acidity, including the "active" acidity and the "reserve" acidity, and this "reserve" acidity remains an unknown quantity made up of undissociated acid molecules as well as the acid held in union by the buffers present in all ordinary nutrient media. Titrating with sodium hydroxide then gives a measure of the total acidity, while the hydrogen-ion concentration measures the true or "active" acidity.

Why is it insufficient to titrate for total acidity? The following illustration taken from Sörensen (II, '09) answers this question. He and other investigators found that certain enzymes, such as invertase, catalase, pepsin, and others, show optimum activity in the presence of a certain amount of acid, but the quantity of acid necessary for this activity could not be definitely ascertained by the ordinary titration method. The reason for this is clear. A solution of an enzyme, such as invertase, contains substances which are capable of combining with acids, so that the optimum acidity depends, for one thing, on the substances, buffers, going with the enzyme. However, without measuring the hydrogen-ion concentration it is not possible to get a measure of the exact

amount of these substances so that it becomes impossible to indicate the optimum degree of acidity which would be the same under all experimental conditions. On the other hand, obtaining a measure of the acidity by means of the hydrogen-ion concentration, it is possible to show that this is constant and is as definite as the nature and quantity of the enzyme. Thus, while the total acidity necessary to give optimum enzyme action is very different for 3 enzymatic solutions, the optimum concentration of hydrogen ions, on the other hand, is the same for all 3.

Using a similar illustration for bacteriological media, it may be said that while the optimum acidity for the growth of a particular organism may be +15 on a medium containing Witte's peptone, it will be different on a medium containing some other peptone. When an organism is found to respond best to a reaction of P_H 7, it will always respond similarly at that reaction no matter what the peptone may be, other things remaining the same. Occasionally it becomes important to know the buffer value of the media and in such cases a determination of the total acidity by ordinary titration is utilized in conjunction with a determination of the hydrogen-ion concentration. Illuminating articles on the buffer values of different peptones have been written by Bronfenbrenner, DeBord, and Orr (II, '21) and by Brown (II, '21).

Titration (Fuller's scale)	P_H
+ 7.5	7.5
+ 8.0	7.4
+10.0	7.5
+11.5	7.1
+12.5	7.4
+14.0	6.8
+14.5	6.8
+15.0	6.9

Not only are different results to be expected because of the difference in principles in titrating for total acidity as compared to a determination of hydrogen ions, but total acidity measurements, such as the procedure involved in calculating Fuller's scale, are rather inaccurate for other reasons. As Clark (II, '15) has stated, the use of phenolphthalein as indicator, titrating when the medium is hot, and the difference in end point used by different investigators due to lack of proper color standards, all tend to make the ordinary titration method variable and inexact.

Since Fuller's scale has had such common usage it seems desirable to present actual comparisons of this scale with the determination of hydrogen ions. Various investigators have given comparative figures, the table given by Norton ('19) and reproduced above, is one illustration of such comparison in bacteriological culture media.

The titrations were made by the ordinary methods using phenolphthalein as indicator. Attention should be called to the fact that figures under titration represent parts per thousand as recommended originally by Fuller instead of parts per hundred as is used by Norton.[1] It will be noted that while the total acidity titration (Fuller's scale) shows the media to be markedly acid, the acidity in terms of hydrogen-ion concentration is very nearly neutral or even alkaline. Figures obtained by me show the same thing. A few are here listed:

	P_H	Fuller's scale
Nutrient beef broth acidulated with 0.1% citric acid	4.8	+24.0
Nutrient beef broth acidulated with 0.05% hydrochloric acid	5.5	+12.0
Nutrient beef broth neutralized with sodium hydroxide	7.0	+10.0
Nutrient beef broth rendered alkaline with sodium hydroxide	7.2	+ 6.0

Here also a medium which is neutral or even alkaline with reference to hydrogen-ion concentration appears acid in Fuller's scale. This of course is not surprising since, as is now well known, the color change of phenolphthalein instead of being at the neutral point, is actually on the alkaline side, between P_H 8 and P_H 9.6, so that media below P_H 8 would appear acid if this indicator is used. It is necessary to emphasize the fact that these comparisons are merely indications of what differences may be expected. Any changes, such as the amount or kind of peptone or of beef extract, will change the value on Fuller's scale even in the presence of the same amount of acid. In these comparisons the medium used consisted of 3 grams of Liebig's beef extract, 10 grams of "Bacto" peptone, and 1000 cc. of distilled water.

GROWTH OF THE FOXTAIL ORGANISM ON VARIOUS MEDIA

Two different kinds of agar were used, the Difco powdered agar and a shredded agar of unknown make. No difference in growth was noted but the first is to be preferred since it is more readily soluble. Difco peptone was used and two different kinds

[1] A discussion of the difference in interpretation of Fuller's scale is given by the writer in another publication (Rosen, II, '22).

of meat extract, dehydrated Bacto beef, and Liebig's meat extract. The dehydrated beef gave better growth but required much longer time to make and resulted in a turbid, dirty product, making hydrogen-ion determinations by indicators very difficult.

Agar Plates.—On peptone-beef agar poured plates, P_H 6.6, kept at 20° to 25° C., colonies appeared within 48 hours. They were white, round, smooth, and in 4 days well-separated surface colonies measured as much as 6 mm. in diameter. At first they showed a bluish tinge but later they became opaque white with a somewhat faintly colored creamy yellowish center. The margin was entire, with a conspicuous colorless zone 2 to 4 mm. wide surrounded by a marked whitish discoloration of the medium which was most conspicuous at the border of the colorless zone and gradually faded out into the color of the surrounding medium. The whitish discoloration might be recognized at a distance of 0.5 cm. from the colorless zone at the end of the fourth day although it was faint at this distance. The colony was somewhat sticky to the touch of a needle but was not viscid; it was glistening, somewhat raised or convex, and internally appeared amorphous, that is, without distinctive markings. As the medium dried out the colonies became indistinct and gradually disappeared from view. Buried colonies made but little growth. On P_H 7.2 peptone-beef agar growth was the same but there was no colorless zone or precipitate.

Agar stroke.—At 25 to 30° C. there was a moderate amount of growth in 48 hours, often reaching a width of around 4 mm. at the base of the slant and tapering upwards. The growth was filiform, raised, glistening, smooth, and opaque white to creamy white, odor was absent or not marked, and the consistency was that of melted butter. Growth was very marked at the base of the slant and in the water of condensation and of syneresis. As the smears became old there was a tendency to form a slight, thin growth at the margin which upon close examination appeared in the form of irregular, fine, thread-like projections. Below P_H 7, a colorless region surrounded the smear and this region in turn was bordered by a dense white precipitate. This precipitate became very marked by the third or fourth day. Above P_H 7 there was no colorless zone or precipitate.

Agar stab.—There was good growth at the surface and slight growth in the upper part of the stab. There was no growth in the lower part of the stab.

Gelatin plates.—On poured plates, kept at about 20° C., testing P_H 6, colonies appeared slowly. In 48 hours they were barely visible, being in the form of fine white points. By the fifth day well-separated surface colonies measured 1.5 to 2.5 mm. in diameter; they were round, the margin was entire, appearing coarsely granular when viewed with the low power of a microscope; the granules were not arranged in any definite pattern but were somewhat larger toward the middle of the colony. The color was glistening white with a bluish lustre towards the margin and a yellowish tinge in the middle. There was no liquefaction and no odor. In contrast to surface colonies, which possess even margins, colonies which were imbedded presented uneven margins, and were often deeply lobed. These colonies appeared very rough under the microscope, and the granules were much larger than those of surface colonies. Imbedded colonies were also much reduced in size. On P_H 7 gelatin plates the growth was the same as on P_H 6 gelatin. As the colonies advanced in age the granulation so evident up to the fifth day gradually disappeared. When plates were kept at 25° C., a temperature which caused a slight softening of gelatin testing P_H 6–7, the growth was noticeably better, well-scattered colonies measuring 5 to 6 mm. on the seventh day; as the gelatin softened the surface colonies sank into the medium and took on concentric striae, a character not evident on firm media. Even after 2 months there was no liquefaction. In contrast to gelatin testing P_H 6.0 and P_H 7.0 there was no growth on gelatin testing P_H 5.5 (P_H 8.2 gelatin refused to gel when it has been rendered alkaline before sterilization). On gelatin growth as a whole was not as good as on agar but it should be noted that this might be due to the comparatively low temperature at which gelatin is kept, the organism being one which grows best at a comparatively high temperature. No colorless zone with white precipitate has been noted on gelatin.

Gelatin stabs.—Growth was visible only in the upper part of the stab. There was no liquefaction.

Plain gelatin stabs.—As there is a possibility that the peptone and beef extract of nutrient gelatin may interfere with the enzymes which liquefy gelatin, stabs were made in a medium containing no nutrients except the 1.5 per cent gelatin. On these the growth was but slight and in the upper part of the stab. Here also there was no liquefaction.

Nutrient broth.—Peptone-beef broth testing P_H 7 at 25° C. produced but a faint surface growth and a slight clouding of the medium in 24 hours. In 48 hours the surface growth was more definite and there was a marked clouding throughout the medium, there was no odor, and there was a flocculent sediment. By the end of the sixth day there was a marked surface growth as well as an irregular, granular growth in the upper part of the medium. On agitation these readily broke up into very finely divided flocculent particles. In old cultures there was a heavy sediment which when agitated rose in thread-like whorls and was broken up with difficulty. (One per cent of peptone was but slightly better than 0.5 per cent.).

Nutrient broth over chloroform.—As compared to growth in nutrient broth in the usual atmosphere, growth in an atmosphere of chloroform was much restricted, there was no surface growth and only faint clouding, but the sediment was the same in amount and kind.

Nutrient broth plus alcohol.—Absolute alcohol was added to nutrient broth in sufficient quantity to make solutions of 4, 5, 6, 7, 8, and 9 per cent. There was no growth in any tube above 4 per cent at the end of 10 days. While this gives no exact information on the actual percentage of alcohol which may inhibit growth, no provision having been made for the volatilization of the alcohol or to account for any combination with substances contained in nutrient broth, it nevertheless indicates the inhibiting action of this substance at relatively low concentrations and is quite comparable to the results obtained by others on non-spore-forming organisms.

Sodium chloride in nutrient broth.—One per cent sodium chloride had no marked influence on growth; with 2 per cent there was a reduction in growth and when 4 per cent was reached there was no growth. In 2 and 3 per cent broth there was a marked tendency to form long streamers which hung down from the surface and broke up slowly when shaken. This character is doubtless in the nature of a response to unfavorable, high, osmotic concentrations and indicates a tendency of the organism to remain united in chains and in clumps under such conditions.

Toleration of acids.—The acids used were acetic, citric, hydrochloric, malic, phosphoric, and tartaric. Various investigators have concluded that the germicidal effect of acids may be due to one or more of the following factors: the hydrogen ions, the

anions, and the undissociated molecules, the last 2 often being considered as important in the action of organic acids. The subject has received considerable attention and good summaries may be had in articles by Chambers (II, '20), Foster and Randall (II, '21), Schoenholz and Meyer (II, '21), and Wolf and Shunk (II, '21, pp. 14–20). It is of course clear that there is very little significance in simply adding 0.05 or 0.1 per cent of the acids employed without ascertaining what the active acidity of the media has become. When this acidity is measured it is then possible to compare fairly accurately the effect of one anion with another, at least at the same hydrogen-ion concentration. The writer has endeavored to follow out the old methods by measuring out 0.05 and 0.1 per cent of acid, adding this to nutrient broth, and then ascertaining by colorimetric comparisons what the hydrogen-ion concentrations are. Comparatively equal amounts of inoculum, 0.1 cc. of suspension from broth cultures 48 hours old, were used in the experiments made in Jena glass tubes, having ascertained that these tubes had no appreciable effect on the P_H value after sterilization. Twenty cc. of medium were used in each tube. The broth was rendered neutral, P_H 7, with sodium hydroxide before sterilization (tests after sterilizing exhibiting the same P_H). The acids were added aseptically after sterilization, the tubes being then incubated for 48 hours before inoculation in order to make certain that contaminations had not occurred.

TABLE III

EFFECT OF ACIDS ON GROWTH, MEDIUM NUTRIENT BROTH

Acid	Per cent	H-ion concentration of medium	Growth at end of 48 hours	Growth at end of 6 days	Growth at end of 9 days
Acetic	0.05	4.9	None	None	None
	0.1	4.8	None	None	None
Citric	0.05	5.0	Good	←	←
	0.1	4.8	None	None	None
Hydrochloric	0.05	5.5(†)	Very good	←	←
	0.1	4.5	None	None	None
Lactic	0.05	5.0	Good	←	←
	0.1	4.8	None	None	None
Malic	0.05	5.0	None	Fair	Good
	0.1	4.7	None	None	None
Phosphoric 85%	0.05	4.9	None	None	None
	0.1	4.3	None	None	None
Tartaric	0.05	4.9	None	Fair (1 out of 3)	←
	0.1	4.5	None	None	None

From table III there is a slight suggestion that acetate and phosphate ions are markedly injurious to growth, while malate and tartrate ions exercise a very retarding influence; in contrast to these the citrate, chloride, and lactate ions have no marked effect. It should be noted that there was no growth in any of the acids tried at the end of 24 hours although neutral broth kept at the same temperature, 25° C., showed marked clouding at the end of that period. This is to be expected since this organism in nutrient broth grows best around the neutral point, between P_H 6 and P_H 8. It is rather surprising to see the phosphate act as a poison and this perhaps needs to be repeated before accepting the result although the work was done carefully and carried out in triplicate. More will be said of this later. As far as the acetate is concerned the result obtained substantiates the findings of others (see, for example, Wolf and Shunk [II, '21, pp. 14–20]).

TABLE IV

CHANGE IN P_H AFTER 10 DAYS GROWTH IN ACID BROTHS

	Original P_H	Final P_H
Citric acid	4.9	7.4
Hydrochloric acid	5.5	7.4
Lactic acid	5.3	6.8
Malic acid	5.0	6.8

Table IV is of interest not only because it shows the production of alkali in this medium, a marked character of this organism, but also because it shows that in a given period of time the final hydrogen-ion concentration is not necessarily the same even though the initial concentration may have been quite similar. It is seen that there are apparently 2 groups, one developing an alkalinity of P_H 7.4, and the other shifting only to P_H 6.8. Why lactic acid should produce a higher final hydrogen-ion concentration than citric acid is a question; perhaps this is due to a difference in the dissociation of salts formed, but in the case of malic acid this is doubtless coupled with a lower amount of growth in the same time period (see table III).

It has already been noted that media will change on standing and that changes may be due to glassware or to some rearrangement in the chemical make-up of the medium. The changes as noted in table V are not very great; yet in some of these it is such that if the hydrogen-ion concentration were the only limiting factor the organism ought to grow in the changed medium.

TABLE V

CHANGE IN P_H OF ACID BROTHS AFTER STANDING 10 DAYS UNINOCULATED

Acids	Original P_H	Final P_H
Acetic	4.9 4.8	5.3 4.8
Citric	5.0 4.8	5.3 5.0
Hydrochloric	4.5	5.0
Lactic	4.8	5.0
Malic	4.7	5.0
Phosphoric	4.9 4.3	5.2 4.8
Tartaric	4.9 4.5	5.3 4.6

For example, citric acid broth changed from P_H 4.8 to P_H 5.0, an hydrogen-ion concentration not sufficient to prevent growth (see table III), yet when citric acid broth testing P_H 5.0, originally P_H 4.8, is inoculated there is no growth. The same is true for lactic, malic, and tartaric acids. Without more experimental data it is not possible to give an acceptable explanation of the change, which in this whole series is toward the alkaline side. There is of course some possibility that even with Jena glass there is a slight amount of alkali produced, particularly in strongly acid media; other possibilities such as the production of compounds in which a greater proportion of OH ions is set free may also be considered.

To test further the effect of the anion of various acids, 0.5 cc. of 0.405 N sodium hydroxide was added aseptically to 20 cc. of such acid media as had previously given no growth. (The alkali had first been sterilized so that there was no need of autoclaving after it had been added.) The results are shown in table VI.

Table VI, in comparison with table III, brings out very sharply the poisonous effect of the acetate anions when the hydrogen ions are not the limiting factors. However, it would appear, judging by the results of the phosphoric acid tests, that there may be limiting factors other than the cation and the anion. For one thing, it is hard to believe that the phosphate radicle, as such,

TABLE VI
ACID BROTHS WITH ALKALI ADDED

Acid	Per cent	P_H after addition of alkali	Growth at end of 10 days
Acetic	0.05	7.0	Good
	0.1	5.7	None
Citric	0.1	6.0	Good
Hydrochloric	0.1	7.4	Good
Lactic	0.1	7.0	Good
Malic	0.1	6.0	Good
	0.05	7.0	Good
Phosphoric	0.1	5.8	Good
Tartaric	0.1	6.2	Good

has any poisonous action in the concentrations used (see account of growth in Fermi's solution). Of course, the undissociated molecule may play the role of poison but this also fails to give a convincing explanation of the action. The specificity of the organism with relation to phosphate may be a factor but this is merely assumption; there may be some chemical rearrangement involved which has not been previously recognized, or the action may be a physical one involving such phenomena as surface tension. Traube (II, '21), for example, believes that surface tension should be considered as entering into the action of acids and bases.

In order to test the effect of autoclaving, acids were added to the medium before sterilizing. Approximately 15 pounds pressure for 20 minutes was used. The following acids were employed: citric, hydrochloric, lactic, malic, phosphoric, and tartaric. They were added to nutrient broth in amounts so as to make 0.1 and 0.2 per cent by volume. In every case the hydrogen-ion concentration, ascertained after sterilizing, was the same in each acid, and at both 0.1 and 0.2 per cent it was P_H 5.2. (The nutrient broth in this case contained dehydrated beef instead of Liebig's beef extract, which was used in the acid experiments previously reported.) No growth was obtained in any of the acid media, either at 0.1 or 0.2 per cent, while the same medium without acid showed excellent growth.

Comparison between cotton and glass wool as filtering agents.— In 1916 Miss Lloyd reported that certain substances necessary for growth of *Meningococcus* were removed when filtering was

done through filter-paper in contrast to glass wool which, when used as a filter, did not absorb these substances. Since the filtering done by the writer, and as commonly practiced, is through absorbent cotton, it seemed desirable to test the effect of glass wool as compared with cotton on the growth of the foxtail organism. Using nutrient agar, adjusted to P_H 7, as the medium no difference in growth was detected, and in another series in which it was desired to obtain a comparison between distilled water and tap water in a medium of nutrient agar each kind of medium was divided into 2 parts, 1 filtered through cotton and the other through glass wool. No difference in growth was evident either when tap water was contrasted with distilled water or when glass wool was used instead of cotton. In still another series, using nutrient agar as the medium, the 2 different filtering agents were used and part of the medium sterilized by the intermittent process in the Arnold sterilizer and the other part sterilized in the autoclave at 15 pounds pressure for 30 minutes. It was thought that if any "growth accessories" or "hormones" were involved a difference in sterilizing temperatures in conjunction with different filtering agents might show a difference in growth, but no difference was observed. It may perhaps be concluded that the foxtail organism does not respond to "growth accessories" although further work is necessary before this is accepted.

Loeffler's blood serum.—Growth restricted, whitish, glistening, filiform, slightly raised, and smooth; the medium was not liquefied.

Lima bean agar slants.—Growth in this medium was excellent; it was fully as good, if not superior, to any other medium tried, and bears out the suggestion made by others of making greater use of vegetable media in the study of bacterial pathogens. (The medium consists of 100 grams of dry lima beans, 15 grams of agar, and 1000 cc. of distilled water.) The beans were cooked until they readily fell apart and were strained through cheesecloth, the cloth being squeezed so that all the available liquid was recovered. (The medium as it finally appeared was rather turbid.) At 30° C. there was marked growth in 48 hours; it was raised, glistening, whitish, filiform, and there was no colorless zone or precipitate (P_H value not tested). As the culture became several days old a slight pinkish color developed in the smear, which was particularly noticeable at the base of the slant.

Corn meal agar slants.—Growth not as good as on lima-bean agar (ingredients in the same proportion as in lima-bean agar); it was filiform, quite the same whitish color as that possessed by the medium itself, and somewhat raised and glistening.

Whey agar slants.[1]—Growth at 30° C., medium testing P_H 5.4, was good; it was raised, filiform, ivory-yellow in color (see Ridgway, Color Standards, 1912), glistening, margin slightly undulating or entire, odor none, consistency slimy in the moist part at the base of the slant, and butyrous, somewhat sticky, in the remainder; there was a tendency to produce small, inconspicuous warts which appeared as whitish dots in a vitreous, ivory-yellow matrix. Surrounding the smear there was at first a colorless zone followed by a white precipitate, but by the fourth day the colorless area disappeared, due to an extension of growth at the margin overgrowing the area. The margin finally appeared markedly white in contrast to the interior, this whiteness being due to the white precipitate which had previously been formed when the smear was narrower. (By carefully removing the bacterial smear it was seen that the white portion coincided with the region where precipitate had been formed.) Growth on whey agar slants testing P_H 7.0 and P_H 7.2 was slightly better than on P_H 5.4 medium, but there was no colorless region and no precipitate. On P_H 9.0 whey agar there was no growth.

Uschinsky's solution.—Very slight growth in 3 days kept at 30° C., medium testing P_H 7.6; at the end of 10 days there was a marked ring with a faint surface growth and the fluid was rendered markedly viscid.

Fermi's solution.—Noticeable surface growth in 3 days which broke up into very fine particles on agitation and left a ring around the tube; there was but slight clouding. At the end of 7 days there was a very marked surface growth and a dense clouding in the upper two-thirds of the medium.

Cohn's solution—No growth.

Potato cylinders.—Growth at 25–30° C. good, glistening, buckthorn-brown (Ridgway), sticky, slightly viscid. After growth of 1 month the cylinders were macerated without difficulty, 10 cc. of distilled water was added to each tube, and, in addition, 1 cc. of potassium iodide. A reddish purple color immediately developed which upon standing for several hours entirely disappeared,

[1]Made up according to the formula given by Miss Bryan (II, '21, p. 149) except that Bacto-gelatin was used instead of Nelson's photographic gelatin, No. 1.

leaving a colorless mass; this was in contrast to uninoculated cylinders and in cylinders upon which *Bacillus Coli* was grown, in which the color remained deep blue.

Nutrient broth plus 1 per cent potato starch.—At the end of 11 days, 1 cc. of alcoholic solution of iodine was added and a reddish blue color developed which disappeared after standing over night. (*B. Coli* growth remained deep blue).

Nutrient agar plus 1 per cent potato starch.—Smears were made across the middle on plates containing nutrient agar plus starch. At the end of 10 days plates were flooded with a potassium iodide solution resulting in a clear zone about 0.5 cm. wide around the smears, while the remainder of the plates were deeply colored. All 3 of the last experiments reported indicate a strong diastatic action.

Potato-dextrose agar slants.—Growth good, white, glistening, not viscid but somewhat sticky, best at the base of the slant, noticeably flatter than on ordinary nutrient agar, margin even, somewhat spreading.

Milk.—By the sixth day, at a temperature of 25–30° C., there was no coagulation and no clearing. By the eighth day there was a color change from light buff to warm buff (Ridgway). The medium was cleared slowly but there was no coagulation and by the end of 8 weeks most of the fluid was clear, clay-colored, with a somewhat slimy coagulum covering the bottom of the tube which, when agitated, broke up into lumps. At the end of 3 months most of the coagulum disappeared.

Litmus milk.—There was no change in color up to the fifteenth day when the medium began to turn blue at a temperature of 25–30° C. (original color a grayish blue). The behavior was otherwise the same as in plain milk.

Methylene blue in milk.—Reduction of the dye was prompt; in 24 hours the blue disappeared from the lower two-thirds of the medium leaving a blue rim above. By the end of 48 hours almost all of the blue had disappeared. Here also there was no curdling, indicating that no lab ferment (chymosin or rennin) was produced. This is further indicated by the fact that gelatin was not liquefied.

Grass decoction agars.—It seemed desirable to test the effect of different grass decoctions on growth, in particular the effect of grass decoction obtained from a susceptible host as compared with a decoction obtained from a resistant host. For this purpose

500 grams of washed, healthy leaves of *Chaetochloa lutescens* and of *C. viridis* were boiled in distilled water for 1 hour, the extract decanted, and the well-cooked leaves squeezed between cheese-cloth in order to obtain a maximum amount of extract. Fifteen grams of agar were added and enough water to make one liter of each decoction. The growth on slants of both of these decoctions was meager and no difference was detected. The experiment indicates that between 2 different cooked decoctions, 1 of a resistant and the other of a susceptible plant, there was no difference in growth, and it is felt that further than this no conclusion seems justified.

Amino acids in relation to growth.—Time has not permitted a thorough study of the role of amino acids in the growth of the foxtail organism, but the experiment here reported is indicative of a line of work that should be fully investigated.

TABLE VII
AMINO ACIDS IN RELATION TO GROWTH

Solution	P_H	Response
(1) 0.1% tyrosine*	4.6	No growth
(2) 0.1% tyrosine + 1.0% dextrose	4.6	No growth
(3) 0.1% tyrosine + beef extract		Noticeable clouding
(4) 0.1% tyrosine + 1.0% dextrose + beef extract		Heavy growth
(5) 0.1% leucine	6.8	Noticeable clouding
(6) 0.1% leucine + 1.0% dextrose		Clouding more pronounced than in (5)
(7) 0.1% leucine + beef extract		Better growth than in (6)
(8) 0.1% leucine + 1.0% dextrose + beef extract		Growth as good as in (13)
(9) 0.1% leucine fraction (alanine, leucine, valine)		Clouding more pronounced than in (5)
(10) 0.1% leucine fraction + 1% dextrose		Marked clouding, better than (6) and (9)
(11) 0.1% leucine fraction + beef extract		Growth about same as in (7)
(12) 0.1% leucine fraction + 1.0% dextrose + beef extract		Growth somewhat superior to (13)
(13) nutrient broth (1.0% peptone + 3 grams beef extract)		Good growth

*The amino acids utilized were kindly supplied by Dr. Barnett Sure.

It should be explained that the amino acids were dissolved in distilled water, and in the case of tyrosine, hydrochloric acid was added in (1) and (2) to aid in dissolution, the amount of acid added being sufficient, as shown by the P_H obtained, to affect growth. The beef extract (Liebig's) was used in the same pro-

portion as was used in nutrient broth, 3 grams per liter. The various solutions were sterilized in the autoclave at about 12 pounds pressure for 20 minutes. The experiment clearly indicates that the foxtail organism not only utilizes amino acids but that growth is influenced by the type of amino acid present, thus the combination of alanine, leucine, and valine yields better growth than leucine alone, although the total amount of amino acid used was the same in both solutions. As compared with peptone (Difco) it is interesting to note that these amino acids in combination with dextrose and beef extract gave just as good a growth, and in case of the leucine fraction, the growth seemed heavier than in peptone media.

Indol production.—Both the sodium nitrite-sulphuric acid and the Ehrlich tests gave negative results at the end of the second, fourth and tenth days. The media used were Dunham's solution, nutrient broth, and one consisting of 1 per cent peptone, 0.5 per cent disodium phosphate and 0.1 per cent magnesium sulphate in a liter of distilled water. (*Bacillus Coli* gave a positive test in Dunham's solution at end of the fourth day, with Ehrlich's test.) It should be noted that according to the recent work of Norton and Sawyer (II, '21) it appears that the old tests, such as sodium nitrite-sulphuric acid, are not reliable.

Nitrite production.—Growth in nitrate broth was fairly good; within 48 hours there was a slight growth on the surface, a marked clouding and a heavy viscid growth in the bottom. Tests for nitrite on the tenth day with starch water, potassium iodide, sulphuric acid gave a deep blue color, indicating the presence of nitrites (uninoculated tubes when tested gave no color).

Hydrogen sulphide production.—Tests with paper saturated with lead acetate hung over nutrient broth and over broth plus potato starch showed no signs of hydrogen sulphide production.

Ammonia production.—Tests with filter paper saturated with Nessler's reagent which was suspended over cultures in nutrient broth showed a reddish brown color, rapidly developing on the paper upon heating the solution, hence, that ammonia was produced.

Gas production.—One per cent of the following substances were used in nutrient broth kept at about 25° C. in fermentation tubes: dextrose, galactose, mannite, lactose, maltose, saccharose, inulin, and glycerin. Growth was very good in all and was sharply limited to the medium contained in the open arm, medium not

being agitated (see pl. 29, fig. 1). No gas was produced in the closed arm in any of the substances listed in contrast to *Bacillus Coli* which showed abundant gas in a number of the solutions. This does not mean that no gas was produced; it has already been shown that ammonia was produced in nutrient broth, but that because of the growth being limited to the open arm, any gas which was produced, and which in this organism must not be noticeable, passed off into the atmosphere through the open arm. However, no carbon-dioxide or hydrogen has ever been collected and it is not likely that they were formed in any appreciable quantity in this organism since it is not an acid producer.

Acid production.—Various experiments indicate that this organism does not produce acid in any of the ordinary media. It has already been noted that in acid broths the medium changes toward neutrality or becomes alkaline and that litmus milk becomes blue. Likewise, in the presence of carbohydrates there is no acid production at any time during the growth period. The following experiments illustrate this. Litmus agars containing no other nutrients than 1 per cent of the following carbohydrates remained blue for 2 months, showing no pinkish tinge at any time: dextrose, galactose, mannite, lactose, saccharose, raffinose, and glycerin. Nutrient agar with brom cresolpurple as indicator containing 1 per cent of the following: dextrose, galactose, lactose, and saccharose showed no trace of yellow in 10 days' growth. Lactose agar stabs with Andrade-Penny's indicator (Holman, II, '14) showed no color, in contrast to *Bacillus Coli* which developed a conspicuous pink color in 48 hours. Instead of producing acid this organism produced alkali in the presence of carbohydrates as is shown in the following table. The media consisted of nutrient broth plus 1 per cent of each of the carbohydrates listed.

TABLE VIII

Type of carbohydrate	Original P_H	P_H at end of 5 days	P_H at end of 10 days
Dextrose	7.2	7.2	7.3
Levulose	7.2	7.2	7.4
Mannite	7.2	7.4	7.4
Lactose	7.2	8.0	8.2
Maltose	7.2	8.0	8.0
Saccharose	7.2	8.0	8.2
Glycerin	7.2	7.2	7.2
Broth (without carbohydrate)	7.2	8.0	8.0

Growth was good in all and exceptionally heavy in dextrose and levulose broth. In every case except glycerin the medium was actually rendered more alkaline instead of acid. Does this mean that the foxtail organism does not utilize carbohydrates? If the production of acid and gas was taken as the only indication of carbohydrate utilization (as some investigators seem to believe) the question would be answered in the negative; yet in the case of the foxtail organism there is evidence to indicate that carbohydrates were used without the production of acid or gas (ammonia production resulted from protein decomposition). It is well known that carbohydrate utilization in complex and in simple organisms commonly results in the production of acid and of gas, but it is also known that certain organisms, such as yeasts, decompose carbohydrates and instead of yielding acid they produce alcohol. There are also definite cases on record where carbohydrates are shown to have been used and alkali produced. Thus Miss Karrer (II, '21, p. 78) states: " . . . . the reaction of all the culture solutions with starch as the source of energy was changed during the growth of the fungus (*Fusarium* sp.), this shift being toward increased alkalinity." She presents numerous curves which show the amounts of starch utilized in nutrient media which were acid at the beginning of the experiment and which developed alkali as growth took place. It is to be noted further that in the nutrient media she employed there was no organic nitrogen.

It is not intended to go into a lengthy discussion of acid and alkali production in relation to carbohydrates, how carbohydrates in the presence of nitrogenous food may first be utilized and thus exercise a "sparing" action on proteins, or how acid and alkali may be formed simultaneously, or how a preponderance of alkali may be formed either because of a preferential utilization of acid radicles or because alkali-yielding substances, such as proteins, are utilized to a greater extent. Considerable work has been done on the subject and much remains to be elucidated. Briefly summarized, the following experiments clearly indicate that carbohydrates are utilized by the foxtail organism; first, the marked diastatic action on starch in various media; second, the good growth obtained on media containing nothing but potato cylinders; third, the slight growth obtained in aqueous solutions containing only 1 per cent of each of these sugars, dextrose, galactose, mannite, and saccharose; fourth, the increased

growth in solutions containing sugar over those in which it is absent, in the amino acid series which was previously presented.

Hydrogen-ion concentration in relation to growth.—It has already been reported that the concentration of hydrogen ions plays an important part in the inhibition or enhancement of growth of the foxtail organism. In addition to the experiment on acid broths, nutrient broth was rendered acid or alkali by adding hydrochloric acid and sodium hydroxide aseptically after the broth had been sterilized. The following series was used: P_H 4.8, 5.0, 6.6, 7.2, 7.4, 7.6, 8.5, 8.6, and 9.8. There was good growth in P_H 6.6, 7.2, 7.4, and 7.6, and no growth in P_H 4.8, 5.0, 8.5, 8.6, and 9.8. This series in combination with the other studies indicate that the optimum reaction lies between P_H 6.0 and P_H 8.0, that the lower limiting reaction for growth is around P_H 5.0, varying somewhat with different media, and that the upper limit for growth to occur is somewhat below P_H 8.5.

NUTRIENTS IN RELATION TO PRECIPITATE PRODUCTION

Production of a colorless zone followed by a white discoloration has already been reported on nutrient agar and on whey agar when these gave a slightly acid reaction. It has also been reported that no precipitate was obtained on nutrient gelatin. In an effort to determine which material in the media contained the ingredient which gave rise to the precipitate a number of experiments were tried. Beginning with agar an attempt was made to substitute other plant materials for the algal product and the gums arabic and tragacanth were tried without success. The first of these when dissolved in water does not give a stiff medium even at very high concentrations, and the second is extremely difficult to dissolve in water. Attempts were then made to thoroughly wash the agar according to the method used by Ayers, Mudge, and Rupp (II, '20). These authors found that unwashed agar contained various calcium and magnesium salts as well as proteins and that by a thorough washing considerable part of these impurities were removed. Shredded agar was used, and after washing the nutrients peptone and beef extract were added in the usual amounts; part of the medium was allowed to remain slightly acid, P_H 6.6, and part was rendered slightly alkaline with sodium hydroxide, P_H 7.2. Good growth was obtained on both, and while no precipitate was obtained in the alkaline medium the characteristic white discoloration occurred on the

acid medium, indicating that impurities contained in the agar were probably not responsible for the formation of precipitate. As agar itself is not ordinarily considered a food, it seems reasonable to conclude that agar was not responsible for the white precipitate.

Water was the next ingredient investigated. Previous tests having indicated that a medium containing tap water, when slightly acid in reaction, gave a heavier precipitate than distilled water, tap water was used. Tap water was therefore treated with strong hydrochloric acid in order to remove carbonates, and boiled for several hours. It was then incorporated into nutrient agar, and part was rendered alkaline, P_H 7.2, and another part acid, P_H 6.6; nutrient agar containing untreated tap water and also 1 part made alkaline and another acid were run at the same time as checks. Growth on the alkaline medium containing treated water was as good as on the medium containing untreated water, but the medium testing P_H 6.6 with treated water did not give as good growth as the slightly alkaline medium. White precipitate occurred in the acid media, both with treated and untreated water. This indicates that water does not influence the production of precipitate. (Chemical tests made of the water at Fayetteville, Ark., by the department of chemistry of the University of Arkansas indicate that of the materials found in the local tap water, carbonates made up by far the largest proportion, other substances being but slightly more than a trace.)

Substitutions were then attempted for peptone; the media used were (1) 1 per cent dextrose-beef extract agar; (2) 1 per cent lactose-beef extract agar; (3) 1 per cent dextrose, and .1 per cent tyrosine-beef extract agar. In all of these the ordinary 0.3 per cent beef extract was used, each divided into 2 parts, 1 rendered slightly acid and the other slightly alkaline. Growth in the first 2 was not as good as in media containing peptone, but the 1 containing tyrosine gave a growth which appeared fully as good as on ordinary nutrient agar. (Davis (II, '17) has found that the value of peptone is governed by the amino acids present and he has concluded that tyrosine and tryptophane are important constituents of satisfactory peptone.) On each of the 3 media listed, precipitate was obtained in the part testing slightly acid. This clearly indicates that the commercial peptone is not responsible for the white precipitate.

Beef extract is the remaining nutrient to be considered. Media containing but 1 per cent dextrose and 0.1 per cent tyrosine in agar were tried, and no precipitate was obtained either in acid or in alkaline media. Growth in these was not good, but this is not the factor in preventing precipitate production since the precipitate was produced in 1 per cent dextrose plus beef extract even though growth was poor. It may then be concluded that beef extract is the probable source of the white precipitate, and while no similar study has been made on whey agar it is quite probable that whey is the source of the precipitate in this latter medium.

CHEMICAL NATURE OF THE PRECIPITATE

The precipitate was soluble in all of the acids tried, including the following: glacial acetic, chromic, citric, hydrochloric, nitric, sulphuric, lactic, picric, and warm boric; it was insoluble in warm or cold water or organic solvents, including methyl, ethyl, and butyl alcohol, glycerin, acetone, ether, petroleum ether, chloroform, oil of turpentine, benzole, xylol, tuluol, benzine, carbon bisulphide, carbon tetrachloride, potassium permanganate, hydrogen peroxide, and ammonium peroxide. This indicates that the precipitate is an inorganic salt. Tests were made for carbonates and phosphates with the result that posphates were clearly detected. Since the precipitate, while conspicuous, may be had in comparatively very small amounts it is rather difficult to test. The procedure was as follows: With a platinum loop the bacterial smear was carefully removed (all tests were made on agar slants); the tube was washed several times with distilled water and care was taken to see that all bacterial growth, where this was a possible factor, as well as particles of medium, were removed. One per cent solution of nitric acid was then used to dissolve the precipitate, the fluid decanted, filtered through fine filter-paper, and tested with ammonium molybdate reagent (see Treadwell's 'Qualitative Analysis'). Tubes containing the same media but without showing any precipitate, the ones reacting alkaline, were treated in exactly similar manner and run at the same time. Many such tests show that the precipitate, if not wholly, is in large part phosphate, as indicated by the yellow color, and precipitate developed in solutions coming from tubes which originally contained the white discoloration.

It is very difficult to obtain a test of any nutrient agar in which the yellow precipitate produced by ammonium molybdate

is entirely absent, since the addition of strong acid may not only disintegrate any bacteria which may be present but may also dissolve some of the nutrient medium. However, there is no difficulty in obtaining a marked quantitative difference of yellow precipitate when the same amount of reagents are used in tubes possessing the white precipitate as against tubes which do not, those tubes containing the precipitate always giving a greater amount of yellow ammonium phosphomolybdate. In a number of tests, where the bacterial growth was removed *in toto*, often very difficult to do without breaking up the medium, and where the acid was allowed to act only a short time, no yellow precipitate occurred in tubes which did not possess the white precipitate.

It seems to be well established that beef extract contains phosphates, the following statement in Eyre's 'Bacteriological Technique,' p. 128, indicating this: "Meat extract . . . is acid in its reaction owing to the presence of acid phosphates . . . ." The following appears to be a plausible explanation for the production of a colorless zone plus a white precipitate on acid media. It has been shown that the foxtail organism in its growth on various media, including nutrient agar, produces alkali. The alkali acts on the acid phosphates and causes them to precipitate out of solution. (It is well known that various acid phosphates are precipitated out of solution when alkali is added.) Immediately around the organism the phosphate is probably used up as growth proceeds, while the phosphate beyond a certain distance of the bacterial growth, not having been used, is acted on by the alkali which diffuses from the region of growth and is precipitated out of solution. There is also the possibility of an action by a carbon dioxide gradient which, being greatest in the region immediately surrounding the colony, might prevent precipitation by the acid reaction. No precipitate is produced on alkaline media, probably because the substances from which the white precipitate is derived were largely precipitated out when the medium was rendered alkaline. This seems to be in keeping with Eyre's observation (p. 150). In giving directions for making nutrient agar he recommends the addition of sodium hydroxide until a reaction of +10 is obtained (this often is equivalent to P_H 7.0 or P_H 7.2 as previously shown) and further recommends the following as the next step after the addition of al-

kali. "Replace in the steamer for twenty minutes (to complete the precipitation of the phosphates, etc.)."

Which phosphates are involved in this precipitate has not been determined. In experimenting with anhydrous disodium glycerophosphate Mellon and his associates (II, '21) decided that this substance acts as a solvent for calcium and magnesium salts. Accordingly, it was incorporated into nutrient agar in the proportion they recommend. The medium was divided into several parts, some being rendered acid and some alkaline by the addition of hydrochloric acid or by sodium hydroxide giving the following reactions: P_H 6.0, 6.3, 6.6, 6.8, and 7.4. Growth on this medium as a whole is somewhat better than on ordinary nutrient agar. It makes a clear medium although a dense precipitate settles at the bottom of the tubes after sterilization. No white precipitate was produced on either the acid or alkaline side. It is of course difficult to say how this is brought about, whether the presence of this salt prevents precipitation by its solvent action on calcium and magnesium salts, or because this salt when added to peptone-beef extract agar precipitates out materials which would otherwise enter into the production of white precipitate, or because of its buffer action in preventing a change in the hydrogen-ion concentration. At any rate, it is quite probable that the white precipitate is in the nature of a complex phosphate and not a simple calcium or magnesium salt.

PHYSIOLOGICAL REACTIONS

Effect of temperature on growth.—The medium used was nutrient broth testing P_H 6.8, care being taken to add approximately the same amount of inoculum, 0.1 cc. of 48-hour broth culture, to each 10 cc. of medium. The organism was subjected to the following temperatures (degrees centigrade): 10, 14, 20, 25, 30, 35, 40°. With the exception of the 30° incubator which maintained very nearly a constant temperature, there was some variation in all of the others amounting to 1–2 degrees, so that the temperatures given are not absolute. In 24 hours there was a heavy clouding at 34–35°, almost as heavy at 30°, noticeably less at 25°, only a slight growth in the upper fourth of the broth at 20°, no visible growth at 14°, and none at 10°. In 48 hours a slight growth was visible at 14°. At the end of a week there was a faint clouding at 10,°, more noticeable clouding at 14°, and beginning with 20° growth was very marked up to 35°, with

none at 40°. Growth appeared first and was heaviest at 30–35°, which may be regarded as the optimum zone. The minimum zone has not been determined but it is probably around 0°, while the maximum is around 40°. Death occurred at 41 to 43°. It is thus seen that the optimum, a rather high temperature, is quite close to the maximum which in turn is close to the thermal death point.

Effect of drying.—Bacterial smears on sterile cover glasses were kept dry for 1 hour, 5 hours, 48 hours, and 3 days, at 25° C. When these glasses were dropped into broth growth occurred in all except the one kept dry for 3 days. One would conclude then from this experiment that the foxtail organism is sensitive to desiccation. The following data indicate that such a conclusion is quite false. Diseased material collected July 30, 1921, and kept in a dry condition in the laboratory at about 25° C. until March 24, 1922, a period of about 7 months, was surface sterilized in the ordinary manner, macerated in sterile water, and sprayed on oat seedlings, giving abundant infections, while a check pot sprayed with sterile water showed none. The experiment was duplicated with the same results.

The writer is convinced that the effect of drying cannot be measured merely by smears on glass, and taken by themselves such tests are apt to lead to erroneous conclusions. The reason for this is apparent, bacteria within infected host tissue being of course more or less closely associated with cellular matter. The action of plant colloids in taking up and holding water has been carefully investigated (see MacDougal, Carnegie Inst. Publ. 297) so that the colloids in association with bacteria must be considered in any water relationships. It thus appears that the foxtail organism is resistant to drying.

Effect of freezing.—Using 48-hour broth cultures, 0.2 cc. was inoculated in tubes containing 10 cc. of broth. Two tubes were placed into a freezing, brine-ice mixture, and 2 into an incubator at 35° C. After 2 hours the 2 tubes, which had remained in the form of solid frozen masses for more than 1½ hours, were removed from the freezing mixture, thawed out, and poured plates made from calculated dilutions. Check tubes kept in the incubator for the same length of time were treated in the same way. Using equal amounts of inoculum no noticeable difference in the number of colonies which developed was obtained. The experi-

ment indicates that the foxtail organism is not readily killed by freezing and thawing.

Effect of sunlight.—Moderately thick sowings of a 48-hour broth culture were made on nutrient agar-poured plates. One-half of each plate was covered with black paper and the plates, resting on ice, were exposed to a bright December sun for 5, 10, 15, and 20 minutes, triplicates being used for each time period. They were then incubated at about 32° C. No difference in number of colonies could be detected for any of the periods in the exposed as against the covered half. The results were rather unexpected since bacterial pathogens are often described as sensitive to sunlight, certainly within the range employed, 5 to 15 minutes being sufficient to kill or very much retard colony development in a large number of forms. The experiment was therefore duplicated in May and exposures of 15, 30, and 45 minutes made to a bright sun. There was hardly more than a 10 per cent reduction in the number of colonies on plates exposed 30 and 45 minutes as compared to those exposed 15 minutes, the difference being within the limits of experimental error. The number of colonies (about 150) which developed after 45 minutes on the exposed half as compared to the covered half was hardly different. The organism does not appear to be very sensitive to sunlight.

Overwintering.—Experiments on the effect of desiccation and freezing indicate that the organism has no difficulty in overwintering. Unfortunately diseased material which had been placed out of doors in the fall to be tested the following spring was destroyed so that there is no direct evidence for overwintering. Field observations as well as laboratory studies indicate that overwintering is readily accomplished. The writer has already recorded the presence of the disease for 4 successive seasons on the same field. The organism may be carried over winter either in diseased glumes, or possibly in the soil in diseased leaves.

Vitality on culture media.—If the substratum is not permitted to dry down, the foxtail organism will continue to live for a long time. In liquid media, such as nutrient broth and milk, growth may be obtained after a period of 6 months, and infections are readily produced from broth cultures kept 4 months at about 25° C. However, when the organism growing on nutrient agar slants is kept in the ice-box for 9 months it loses its viability.

Group number.—According to the most recent chart adopted by the Society of American Bacteriologists (Conn, et al, II, '20), the foxtail organism has the following index number: 5322-31220-1333.

Technical Description

Pseudomonas alboprecipitans,[1] n. sp.[2]

Narrow rods with rounded ends, solitary or in pairs; average measurement of single rods 0.6 by 1.8 μ, motile by a single polar flagellum; no spores, zoogloea or irregular forms; capsules present; strict aerobe; surface colonies on nutrient agar white, round, somewhat raised, smooth, amorphous, sticky, margins entire, surrounded by areas followed by a white precipitate on media testing acid, as P_H 6.6; nitrates reduced to nitrites; ammonia produced; indol and hydrogen sulphide not produced; no acid or gas produced in the presence or absence of carbohydrates; diastatic action strong; no growth in Cohn's solution but growth in Uschinsky's and Fermi's solutions is fair; minimum temperature about 0° C., optimum between 30 and 35°, maximum about 40°, thermal death point 41–43°; vitality on culture media comparatively long; not sensitive to drying or freezing; not very sensitive to sunlight; Gram-negative, not acid fast. Pathogenic to *Chaetochloa lutescens, C. geniculata, C. italica, Avena sativa, Holcus Sorghum, H. Sorghum sudanensis, Hordeum vulgare, Secale cereale, Triticum sativum,* and *Zea Mays.*

Summary

The bacterial disease here described is common on yellow foxtail in Arkansas. No concerted effort has been made to discover the disease on other grasses but artificial inoculations show that the pathogen is infectious on wheat, oats, rye, barley, corn, Sudan grass, millet, and perennial foxtail.

[1]According to the classification suggested by Smith (II, '05, p. 171) the combination would be *Bacterium alboprecipitans* n. sp.

[2]**Pseudomonas alboprecipitans,** sp. nov., aerobus; baculis asporis cylindricis singulis vel binatis apicibus utrinque rotundatis flagello uno polare mobile, baculis solitariis 0.6×1.8 μ.

Coloniae in agar-agar rotundatae albae leves, marginibus plenis; in culturis acidi minimi zonae hyalinae factae sunt secutae a precipito albo. Gelatina non liquefacit. Acidum et gas non efficiuntur.

Habitat in foliis vivis *Chaetochloae lutescentis, C. italicae, C. geniculatae, Tritici vulgaris, Secalis cerealis, Avenae sativae, Hordei vulgaris, Zeae Mays, Holci Sorghi.*

Arkansas, Amer. bor.

A large number of varieties of various cereals are subject to attack. Artificial inoculations also indicate that the organism is capable of doing serious damage, particularly to seedlings of oats and barley.

Lesions on foxtail appear as light brown or dark brown spots and streaks of no definite size or shape and may occur on any part above ground, but are most often found on blades and sheaths.

Attacks on other hosts vary from light yellow indefinite areas, often with a reddish tinge in the case of oats, to grayish-green, markedly withered areas.

The disease appears to be different from any other known bacterial disease of grasses.

Attacked tissues teem with bacteria which discolor, disintegrate and finally kill the invaded tissue. Entrance to the host is by means of stomata and water pores.

The organism is a single-flagellate rod, white in culture, with colonies surrounded by a characteristic colorless area followed by a white precipitate on slightly acid media. It is described as *Pseudomonas alboprecipitans* n. sp.

The meaning of hydrogen-ion concentration, its relation to titratable acidity, methods of measuring it, and the necessity of utilizing it in the study of bacterial pathogens are discussed. Comparisons are given between Fuller's scale and P_H values.

Numerous cultural reactions are presented, including a method for definitely controlling precipitate production by means of varying the hydrogen-ion concentration; the relationship of various organic anions to growth, comparison between cotton and glass wool as filtering agents, the use of lima bean agar in bacterial studies, and a study of several amino acids in relation to growth of bacteria are also discussed.

It is found that beef extract is the probable source of the white precipitate in media which contain the extract and that this precipitate is a phosphate.

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Explanation of Plate

PLATE 23

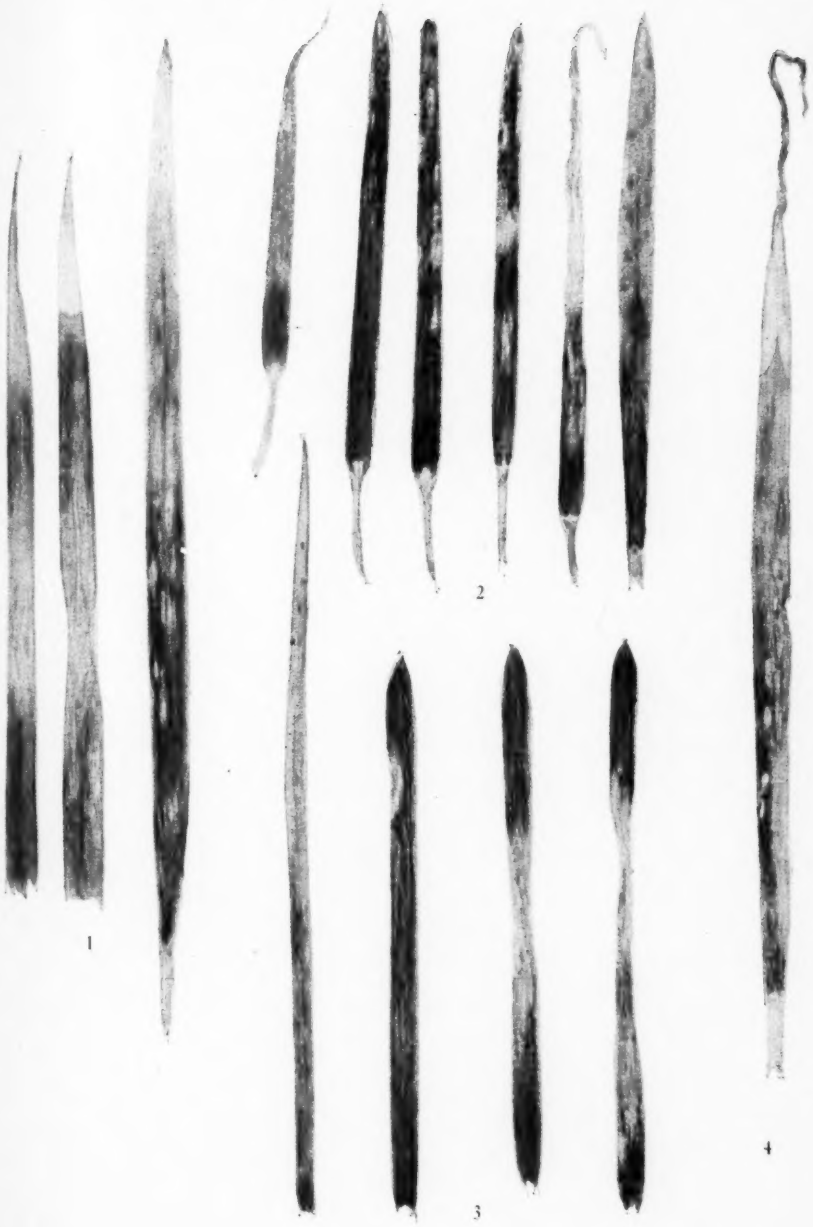
Fig. 1. Natural infections on foxtail (3 leaves).
Fig. 2. Artificial infections on foxtail (2 leaves).
Fig. 3. Artificial infections on sorghum, variety "Amber Cane."
Fig. 4. Artificial infections on *Chaetochloa geniculata*.
Fig. 5. Artificial infections on corn.

ROSEN—A BACTERIAL DISEASE OF FOXTAIL

Explanation of Plate

PLATE 24

Fig. 1. Artificial infections on barley (3 leaves).
Fig. 2. Artificial infections on oats (6 leaves).
Fig. 3. Artificial infections on wheat (4 leaves).
Fig. 4. Artificial infections on rye.



ROSEN—A BACTERIAL DISEASE OF FOXTAIL

Explanation of Plate

PLATE 25

Upper figures represent various types of natural infections on foxtail, including blackish streaks and blotches, small dark brown, oval-shaped or roundish spots with light centers and withered light brown tips.

Lower right-hand figures represent artificial infections; left-hand figure a normal head of foxtail (*Chaetochloa lutescens*).

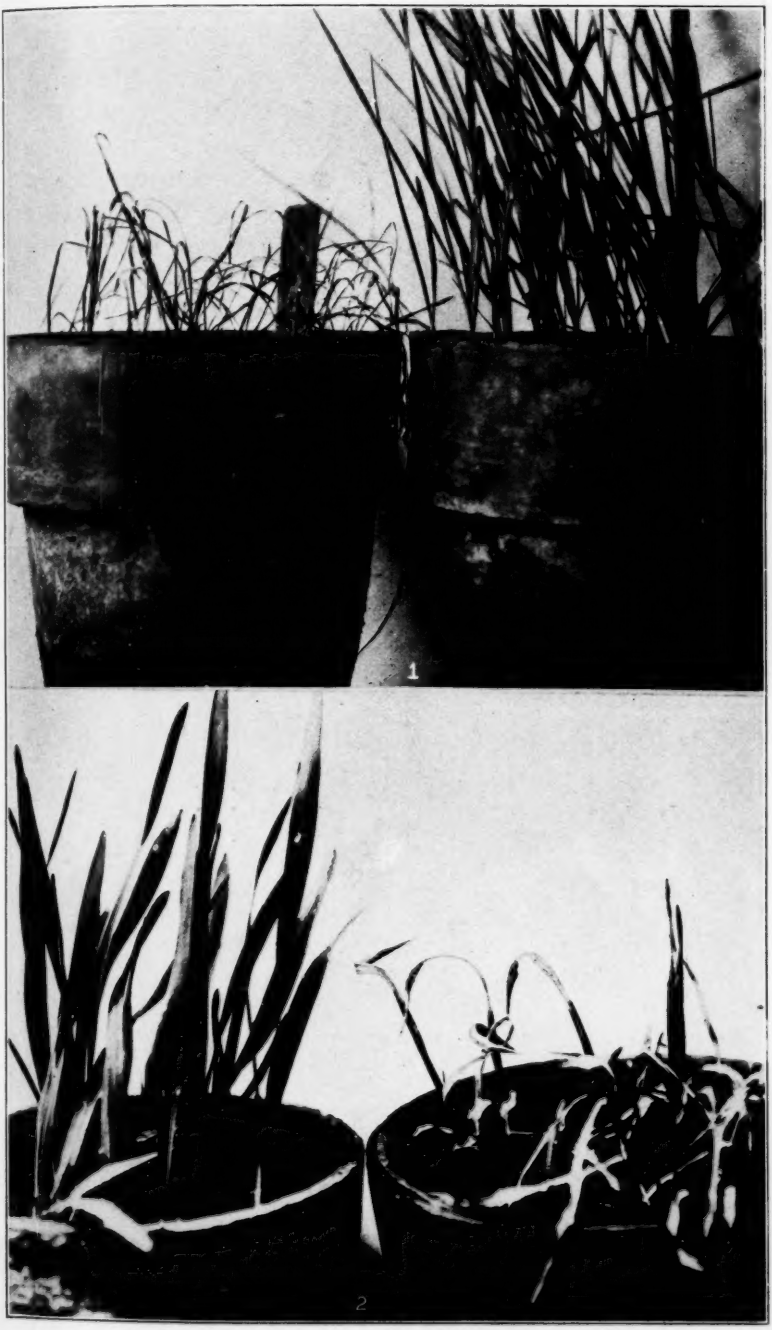
ROSEN—A BACTERIAL DISEASE OF FOXTAIL

Explanation of Plate

PLATE 26

Fig. 1. Pot on the left—dead oat plants 4 days after inoculation with the foxtail organism; on the right—check plants of the same age sprayed with sterile water.

Fig. 2. On the right—badly diseased barley seedlings 4 days after inoculation; on the left—barley plants of the same age sprayed with sterile water.



ROSEN—A BACTERIAL DISEASE OF FOXTAIL

Explanation of Plate

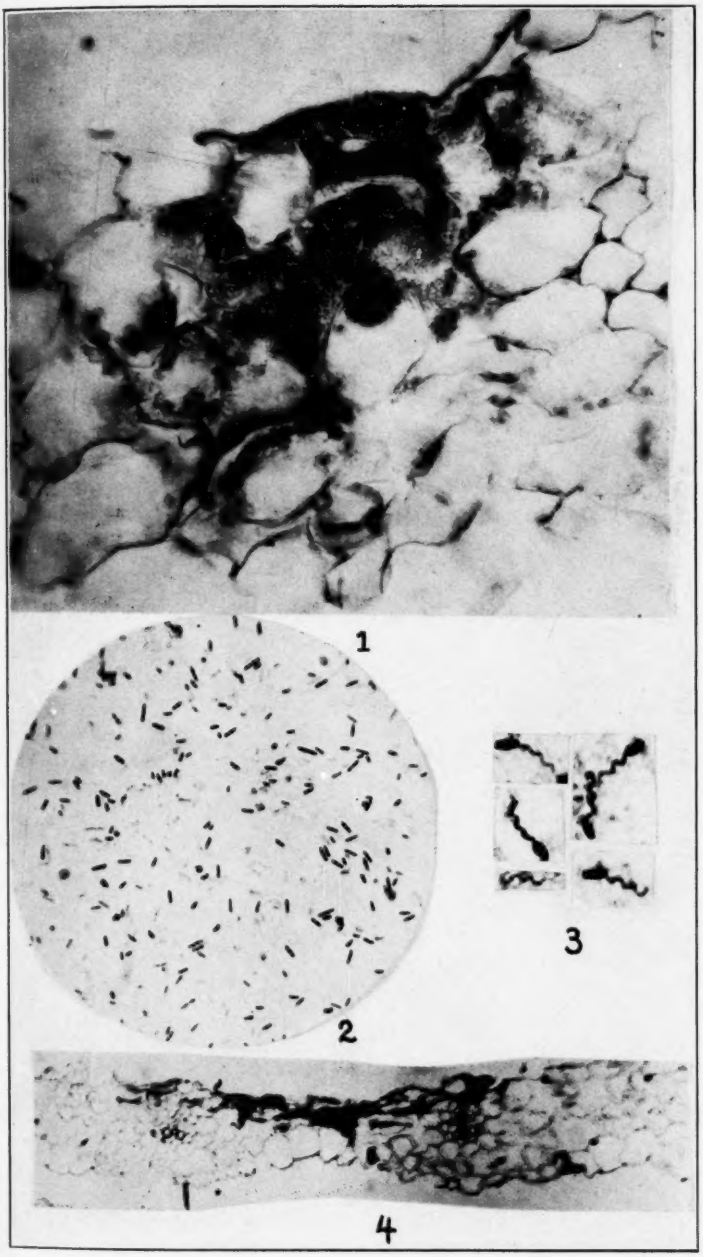
PLATE 27

Fig. 1. Section through a diseased spot of an oat leaf artificially infected, showing portion of a substomatal cavity as well as neighboring cells and intercellular spaces filled with bacteria. Magnified about 950 times.

Fig. 2. Foxtail organism stained with carbol fuchsin, obtained from young agar slant culture. Magnified about 1000 times.

Fig. 3. Foxtail organism stained with Van Ermengem's flagella stain. Magnified about 1200 times. Bodies of the organisms, except the lower ones to the left, retouched; all flagella remain untouched.

Fig. 4. Cross-section of a diseased oat spot showing discoloration and collapse of attacked cells. Magnified about 450 times.



ROSEN—A BACTERIAL DISEASE OF FOXTAIL

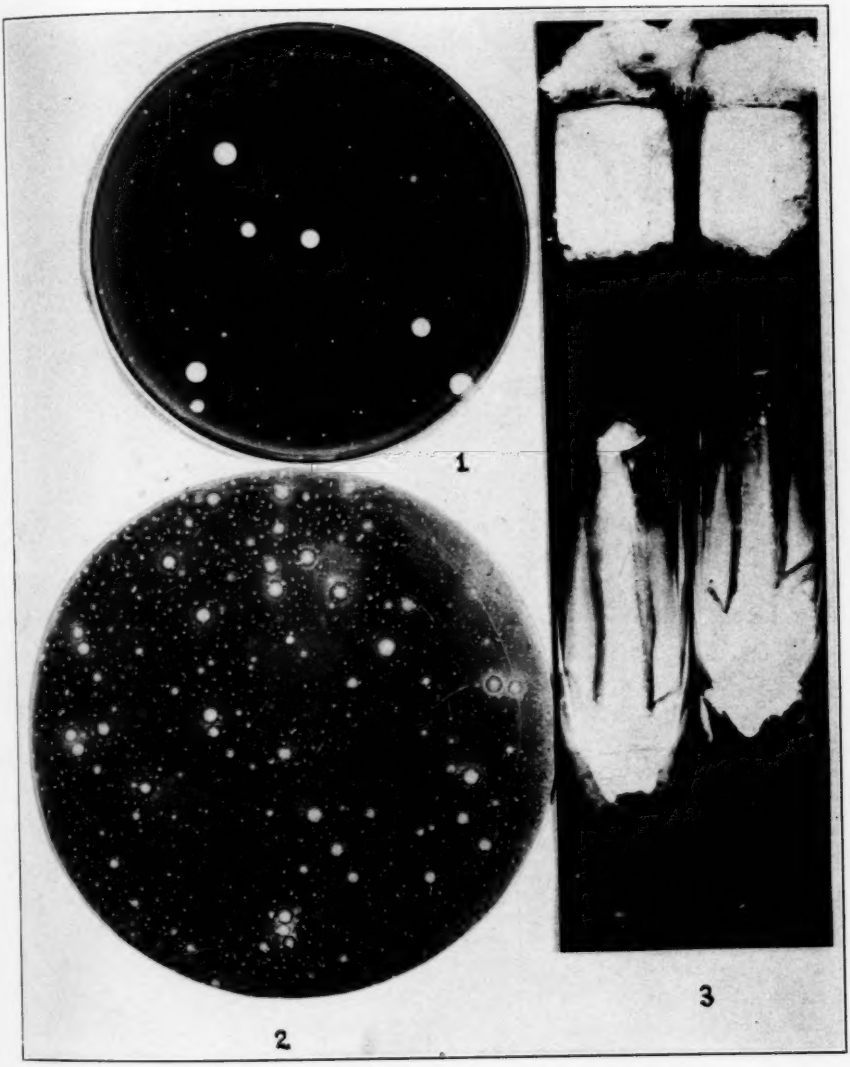
Explanation of Plate

PLATE 28

Fig. 1. Four-day colonies on nutrient agar testing P_H 6.6, showing colorless zone surrounded by a marked white precipitate.

Fig. 2. Same as fig. 1, showing a thickly sown plate with a coalescing and spreading grayish sheet of colonies deeply imbedded and growing next to the glass.

Fig. 3. Ten-day slant of nutrient agar testing P_H 6.4 showing colorless zone surrounded by a marked white precipitate.



ROSEN—A BACTERIAL DISEASE OF FOXTAIL

EXPLANATION OF PLATE

PLATE 29

Fig. 1. Dense clouding in open arm in 1 per cent mannite-nutrient broth with no growth above the arrow point, and no gas.

Fig. 2. Wheat leaves inoculated at infected points with a pure culture of the foxtail organism, by means of a sterilized platinum loop.

1

2

ROSEN—A BACTERIAL DISEASE OF FOXTAIL

THE TOXIC PROPERTY OF SULPHUR[1]

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INTRODUCTION

Since the introduction of spraying for the control of parasitic fungi there has been developed a large number of fungicidal mixtures. Some have proved effective for the control of one organism and some for another, none of them having universal fungicidal value. Because of its abundance, low cost, and its effectiveness under certain conditions, sulphur has been employed in many of these mixtures. The fact that it has toxic or fungicidal properties has been conclusively demonstrated. In this work, an attempt has been made to determine if possible the exact nature of this fungicidal property, that is, to determine or evaluate the chemical compound or compounds in which this toxic property is resident, at the same time to relate this toxic property to conditions under which it may be consistently manifest, thus warranting its general use as a fungicide.

The use of sulphur as a fungicide probably antedates that of all other substances. The chemical and physical properties of sulphur, especially its existence in so many forms, have led to its employment as a fungicide in a variety of ways. Regardless of the form in which it is employed, whether as a compound or as uncombined sulphur, there seem to be necessary certain chemical or physical changes before its toxic properties are exhibited. Toxicity has been attributed to many of the forms, for example, to such products of combined sulphur as various sulphides, thiosulphates, sulphur dioxide, sulphuric and sulphurous acids, and also to uncombined sulphur as flowers, or even as sulphur in a more finely divided state, that is, as colloidal sulphur. However, there seems to be no tangible evidence in the past work that toxic properties can be attributed directly to any one of these forms, the presence of which might thus determine its value as a fungicide. The exact state or states in which sulphur is toxic was left as a matter of considerable speculation.

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment of the requirements for the degree of doctor of philosophy in the Henry Shaw School of Botany of Washington University.

[2] A fellowship established by the Crop Protection Institute for the investigation of sulphur as a fungicide.

History

The generally employed sulphur sprays, namely, flowers of sulphur and the various sulphide compounds, have been only partially effective in controlling fungous diseases. It will not be necessary in this paper to go into a historical discussion of the effectiveness of these sprays, as such discussions are reported frequently by agricultural experiment stations and horticultural societies in bulletins and spray calendars and my own conception of the practical problems involved will be stated below. This work has to do largely with the fungicidal properties of sulphur.

The toxicity of the flowers of sulphur has been attributed to several compounds, of which hydrogen sulphide, sulphur dioxide, sulphurous and sulphuric acids, and volatile sulphur are more often given. Pollacci ('07) believes that sulphur is transformed into sulphuretted hydrogen, the vapors of which have a very energetic action on the fungi. This view, however, has received but little support and has been proved untenable by Bourcart ('13). He was unable to collect any of this gas on passing air from sulphur through solutions suitable for retaining the gas. Foreman ('10) could obtain no inhibition of germination with spores of *Botrytis cinerea*, using a saturated solution of sulphuretted hydrogen. Similar results were obtained by Barker, Gimingham, and Wiltshire ('20). It is at present generally accepted that hydrogen sulphide is not a factor as a fungicidal property of sulphur.

The view that the toxic action of sulphur is due to sulphur dioxide has received considerable support. Sostegni and Mori ('90), Blodgett ('13), Butler ('17), and Kuhl ('21) conclude that the toxic property of sulphur is due to this gas. They believe that the gas is slowly produced by the oxidation of the sulphur. In the papers cited there is little substantiating experimental evidence, other than the fact that the toxic compound is volatile. Contrary views are held by Bourcart ('13) who states that "Sulphurous acid must not be dreamt of; 1/40,000 of this acid in the air would burn the leaves." In a series of experiments he could not collect any sulphur dioxide evolving from sulphur at temperatures up to 50° C. Barker, Gimingham, and Wiltshire ('20) obtained good germination of spores of *Nectria ditissima* in a 1:100 solution of sulphur dioxide. Closed-ring experiments, however, gave limits of .005 per cent and .0005 per cent for the germination of spores of *Sclerotinia fructigena, Fusicladium den-*

driticum, F. Pyrinum, Botrytis cinerea, and *Nectria ditissima.* They conclude that sulphur dioxide cannot be a factor.

Marcille ('11) attributed the toxic property of sulphur to sulphur trioxide and sulphuric acid in the control of grape mildew. A similar conclusion was arrived at by Moissan ('04) who was able to obtain this gas from the spontaneous oxidation of different kinds of sulphur at ordinary temperatures. As far as the author is aware, these results have never been confirmed, and Bourcart ('13) and Barker, Gimingham, and Wiltshire ('20) proved on the contrary that sulphur trioxide and sulphuric acid do not contribute to the fungicidal property of sulphur.

That sulphur is toxic because of its volatilization as such is probably the view most commonly held at the present time. The fact that spores are inhibited in germination when not in direct contact with the sulphur particle has been frequently demonstrated. Smith ('06), working with asparagus rust, concluded that sulphur acts by its fumes but that the sulphur must be uniformly distributed to be effective in controlling the disease. He found that the disease was best controlled in air pockets which aided in preventing a too rapid spreading and dilution of the fumes. Similar views are held by Mares and Mohr (see Bourcart, '13), Bioletti ('07), Bourcart ('13), Barker, Gimingham, and Wiltshire ('20), Doran ('22), and others.

The conditions under which sulphur is volatile or under which volatile substances are formed from sulphur have been investigated by Marcille ('11), Bourcart ('13), Blodgett ('13), Kuhl ('21), and Doran ('17, '22), with the following general conclusions: (1) a certain temperature must be maintained, usually above 25° C; (2) oxygen is necessary; (3) sunlight is a possible factor; (4) the influence of the leaves and spores is considered by some a factor. These conclusions were arrived at by the use of flowers of sulphur.

The toxicity of other forms, such as finely divided sulphur and the various sulphides, has been investigated by a number of workers. Doran ('22) found that Schloesing's precipitated sulphur[1] was more effective in killing spores of *Venturia inaequalis* than any of the finely divided sulphurs used. Atomic sulphur[2] has been reported effective.

[1] Manufactured by Usines Schloesing Freres et Cie., of Marseille, France.
[2] Prepared by the General Chemical Co., New York and Baltimore.

Since the origination of lime sulphur as an orchard spray by M. F. Dusey of Fresno, California, in 1886, there has been a number of studies made on its effectiveness as a spray and on its chemical composition. The first of these of importance was by Thatcher ('06). He found that lime sulphur contained for the most part calcium polysulphides, calcium thiosulphate, and small quantities of sulphites and sulphates. Haywood ('09), using the same methods, obtained similar results. When he dried the mixture the polysulphides disappeared and increasing amounts of precipitated sulphur were formed. He attributed the fungicidal value of lime sulphur particularly to the thiosulphates and possibly to a combined or a summation of the toxic properties of all the compounds formed exclusive of sulphur.

Van Slyke, Bosworth, and Hedges ('10) made some chemical determinations of lime sulphur when the ingredients were varied. They came to the conclusion that a mixture containing a high proportion of sulphur had the largest amount of calcium pentasulphides and a greater fungicidal value. They proposed the following formula: 80 lbs. sulphur, 36 lbs. calcium oxide, and 50 gallons water. This formula is the one in general use at the present time. Their chemical determinations gave about the same results as those obtained by Haywood ('09). Ruth ('13), in a study of lime sulphur and lead arsenate mixtures, found that no arsenic sulphide was formed. The proportion of thiosulphates and sulphites was increased in this mixture, and he attributed the increased effectiveness of the spray to the presence of additional quantities of these compounds. There was no experimental evidence for this, and his chemical determinations did not show the presence of any particular toxic compound. Harris ('11) made chemical determinations of lime sulphur mixtures and found about the same amounts of sulphides, sulphites, etc., as Haywood. He also stated that filtering was unnecessary. Official methods for the determination of the compounds formed in lime sulphur are given by Roark ('20) and Winter ('20).

The above studies on lime sulphur have had to do with freshly prepared mixtures. Vermorel and Dantony ('19) gave a number of reactions that probably took place in lime sulphur mixtures and the compounds formed when the mixture was aerated. They found that the polysulphides soon disappeared after the spray was applied. The calcium thiosulphates gradually decreased, and sulphites, sulphates, and free sulphur increased. Barker,

Gimingham, and Wiltshire ('20) concluded that calcium thiosulphate, hydrogen sulphide, and sulphur dioxide were all slightly toxic but not sufficiently so to account for the fungicidal value of lime sulphur. The calcium pentasulphides were toxic, but since they disappeared in a few hours the lasting toxicity of lime sulphur could not be attributed to them. They concluded that the lasting toxic property must be due to precipitated sulphur. Doran ('22) also found that the sulphides decomposed very rapidly, especially when dried slowly.

Several other sulphide preparations have been employed as fungicides but have proved more or less ineffective as a lasting spray because their retention on the tree as sulphides is difficult to maintain. Their caustic nature frequently results in severe burning.

In testing the toxicity of sulphur and its compounds considerable confusion has developed owing to the variation in resistance of different species of fungi. Barker, Gimingham, and Wiltshire ('20) found that germination of the spores of *Sclerotinia fructigena* and *Phragmidium subcorticium* were entirely inhibited in a suspension of flowers of sulphur in Van Tieghem cells. Germination of *Fusicladium dendriticum* and *Cladosporium fulvum* were 50 per cent inhibited, while that of *Nectria ditissima, Botrytis cinerea,* and *Verticillium* sp. was not at all inhibited. When the flowers of sulphur was used Doran ('22) found that a much higher temperature was necessary for the killing of spores of *Botrytis cinerea* than for *Venturia inaequalis* and a higher temperature for the latter than for spores of *Sclerotinia cinerea.*

Experimental

Since most of the evidence listed in the foregoing references points to sulphur as being the toxic agent regardless of the sulphur mixtures used, it was first thought important to study the influence of the sulphur particle and molecule on the germination of spores. The Van Tieghem cell and the hanging-drop culture method, later slightly modified, were employed. The percentage of germination of the spores was used as an indication of toxicity. The organisms used were selected from the group of strict parasites most of which are of economic importance. It was also necessary to select those that sporulated readily. The following forms were used: *Colletotrichum Gossypii, Sclerotinia cinerea, Botrytis cinerea, Glomerella cingulata, Gloeosporium*

venetum, Macrosporium sarcinaeforme, Phomopsis Sojae, and *Actinomyces Scabies.* These organisms were grown on potato agar prepared according to the method of Duggar, Severy, and Schmitz ('17), and spores were taken from cultures 10–15 days old.

The culture solution used in the hanging drops and in which the sulphur particles were suspended was a slightly buffered mixture containing mannite, phosphoric acid, and sodium hydroxide. The solution was prepared according to the method of Karrer and Webb ('20), as follows: Stock solutions of M/5 mannite in M/10 phosphoric acid and M/5 mannite in M/5 sodium hydroxide were made. Equal quantities of the M/5 mannite-M/10 phosphoric acid were placed in each of 10 flasks and successively increasing proportions of M/5 mannite-M/5 sodium hydroxide were added. The flasks were plugged with cotton, sterilized at 15 lbs. pressure for 15 minutes, and allowed to stand for a few hours. Titrations made by the colorimetric method (Clark, '20) showed the mixtures to have the following range of hydrogen-ion concentrations: P_H 1.6, 2.4, 3.2, 4.2, 5.2, 5.8, 6.4, 6.8, 7.4, 8.4.

EXPERIMENT I. TOXICITY OF THE FLOWERS OF SULPHUR

Since sulphur in the form of flowers is insoluble in any solution that can be used for the growing of fungi, it was necessary to test its toxicity in the form of suspensions. Twenty test-tubes were provided with pipettes that extended through the cork stoppers and to the bottoms of the tubes. By this means drops could be transferred readily to the hanging-drop cells. These test-tubes constituted a duplicate series of 10 each, and 5 cc. of each of the slightly buffered solutions were added to the tubes so that each tube represented a particular hydrogen-ion concentration. To one series .5 gm. of flowers of sulphur was added to each tube.

The technique of the planting of the hanging-drop cultures was essentially the same as that used by Webb ('21) in his germination studies, and was as follows: Ground glass rings were cemented to glass slides by means of parawax and petrolatum. Two of these rings were placed on each slide, and 20 slides constituted a series for each organism. This gave duplicate cultures for each hydrogen-ion concentration. A few drops of the sulphur suspension to be tested for its toxicity were placed in the bottom of the two cells. Another drop was placed on a clean

sterile glass slide. A loop-full of spores was placed in this drop and the spores evenly distributed throughout the drop. By means of a small sterile glass rod a small portion of this drop was transferred to a clean sterile cover glass and the latter inverted over the glass cell. The cell was made air-tight by sealing with petrolatum. In like manner cultures were made representing each hydrogen-ion concentration both with and without sulphur. The series of hanging-drop cultures were then kept at room temperature. Examinations were made at the end of 16 and 24 hours.

After examining some of the preliminary cultures it was found that considerable irregularity in germination existed. Some types of spores would remain on the surface of the drops and often would not be in close proximity to the sulphur. Other types of spores were found to be in the center of the drops with the sulphur particles. Different-sized drops would often result in irregular germination in the control cultures. With some organisms the number of spores in the drop influenced the rate of germination. To eliminate such chance for error a definite spore suspension was made and the drop on the cover glass was spread over a much larger surface, giving it more the nature of a smear. In this way a more even distribution of both the spores and the sulphur particles was obtained. The results of the experiment are recorded in table I and figs. 1–4.

Sulphur in this form was found to be directly toxic to only two of the organisms used. In the other forms the spores were not only not inhibited from germinating but the germ tubes grew normally when in direct contact with the sulphur particles. It can only be concluded from these results that if the flowers of sulphur has a general fungicidal value it must be due to some change in the form of sulphur and that this change takes place under different conditions than were obtained in closed-ring Van Tieghem cells. Within the usual range for germination the hydrogen-ion concentration influenced the results but slightly.

EXPERIMENT 2. FINELY GROUND FLOWERS OF SULPHUR

Since the ordinary flowers of sulphur was toxic to two of the organisms, it was concluded that there was a toxic property present but in a very dilute form. If physical conditions influenced the production of this property it was thought that possibly a finely ground product might be more effective. To obtain sulphur in this state an electrically driven excentric mortar, as used for

crushing yeast cells, was employed. Eight gms. of flowers of sulphur were mixed with 3 gms. of diatomaceous earth (Kieselguhr), and the mixture ground for 14 hours. One-half gm. of this mixture was added to each test-tube containing the slightly buffered solution of the different hydrogen-ion concentrations and the toxicity determined as before. An attempt was made to grind the sulphur without the diatomaceous earth but the sulphur had a tendency to cake and did not grind well. Other substances are being tried with the hope of eliminating diatomaceous earth. Results of this experiment are given in table 1, figs. 1–4.

Sulphur in this state was found to be more toxic than the flowers of sulphur unground. A more marked influence of the hydrogen-ion concentration was noted, the range showing the greatest toxicity being between P_H 4.2 and 5.4. The increased toxicity at this point is attributed to one of 2 possibilities: first, the spores may be less resistant at this point, or second, the toxic form or conditions of sulphur may have been produced in greater amounts at this range. At any rate the hydrogen-ion concentration and the fineness of the particle contributed to the increased toxicity of the sulphur. The fineness of the particle did not seem to be the direct cause, as germ tubes grew normally after the initial retardation, even though they were directly in contact with the sulphur particles.

EXPERIMENT 3. COLLOIDAL SULPHUR

Sulphur readily assumes the colloidal state. The element sulphur has been known since the beginning of history, and records show that colloidal sulphur was prepared and studied as early as the seventeenth century. "Lac Sulfurus," a colloidal form of sulphur, was prepared in 1765 by Stahl (1766) and was used at that time for medicinal purposes. Fourcroy (1790), Berthollet (1798), Berzelius (1808), and Magnus (1827) were early contributors to the study of colloidal sulphur. Present-day methods for the preparation of colloidal sulphur are found in papers by Svedberg ('09), Himmelbauer ('09), Raffo ('08, '11), Odén ('13), v. Weimarn and Molyschew ('11), Kelber ('12), and others.

Colloidal sulphur exists in two forms, depending upon the degree of hydration. The form having a very high degree of hydration will be discussed in this paper as the hydrophilic colloidal sulphur and is identical with the product prepared by Raffo and Mancini ('11) and Odén ('13) and called "soluble

colloidal sulphur." The other form of colloidal sulphur is that first prepared by v. Weimarn and Molyschew ('11). This last has a very low degree of hydration and will be designated in this paper as hydrophobic colloidal sulphur. A more detailed description of these forms will be given in a subsequent section.

The hydrophilic colloidal sulphur was prepared according to the methods of Raffo and Mancini ('11) and Odén ('13) with certain modifications. Fifty gms. of pure crystalline sodium thiosulphate were dissolved in 30 cc. of distilled water; 70 gms. of concentrated sulphuric acid, sp. gr. 1.84, arsenic free, were weighed into a glass cylinder of 300 cc. capacity. The cylinder was placed in a vessel of cold water and the saturated solution of sodium thiosulphate added very slowly with occasional stirring. The mixture was then allowed to cool and 30 cc. of distilled water added. It was then placed on the water bath and warmed at 80° C. for 10 minutes, and filtered through glass wool to remove insoluble sulphur. The filtrate was cooled and allowed to stand for 12 hours. It was again warmed, filtered through glass wool, and the filtrate cooled. This warming, filtering, and cooling was repeated until no more insoluble sulphur came down. The final filtrate was a slightly turbid yellowish solution. This was centrifuged for 30 minutes at 1500 revolutions per minute. A portion of the colloidal sulphur was thrown out of solution. The supernatant liquid was a clear yellowish solution and was saved for further purification. The residue was washed in cold distilled water and again centrifuged for the same length of time and at the same speed. The supernatant liquid was again yellowish and was saved. The washing and centrifuging of the residual colloidal sulphur were repeated until the residue peptized in water gave a reaction of P_H 4.2. This colloidal suspension was faintly yellow and upon standing 1 week some of the particles settled out, the solution retaining its faint yellow color. Upon drying and weighing, the suspension gave a percentage of sulphur of 3.4.

The supernatant liquids collected from the above were treated with a concentrated solution of sodium chloride, whereby the yellowish colloidal sulphur was coagulated. The sodium chloride was added until no more coagulum seemed to form. The coagulum was easily centrifuged out and repeptized in 10 cc. of distilled water. The color of this solution was a deeper yellow and only very slightly turbid. This colloidal suspension gave a reaction

of P_H 4.2 and did not settle out on standing for 2 months. On drying and weighing, the solution was found to contain 1.6 per cent sulphur. This latter preparation was a typical hydrophilic colloidal sulphur and was more nearly a true "soluble" sulphur than the product obtained from the method of Raffo and Mancini ('11). The first preparation was a mixture of hydrophilic and hydrophobic colloidal sulphur. Odén ('13), in a detailed study of this type of colloidal sulphur mixtures, found them to contain particles of different sizes ranging from the molecular to particles easily discernible under the low power of the microscope. He was able to obtain suspensions with particles varying from the smallest to the largest by fractional coagulation with sodium chloride. Particles of larger size were more easily coagulated than the smaller ones. In colloidal sulphur suspensions of this kind the particles have a tendency to collect themselves into groups, forming larger particles which settle out rapidly. The smaller the particles the slower this takes place and in hydrophilic colloidal sulphur suspensions only a small amount of settling out can be noted after several months.

The chemical reactions involved in the formation of colloidal sulphur prepared by this method is given by Odén as follows:

$$Na_2S_2O_3 + H_2SO_4 = Na_2SO_4 + H_2S_2O_3$$
$$H_2S_2O_3 = SO_2 + H_2O + S$$
$$2\,H_2S_2O_3 = 2H_2S + 2SO_2$$
$$2\,H_2S + SO_2 = H_2O + S$$

$$3H_2S_2O_3 = 3H_2O + H_2SO_4 + S + S$$

Further chemical reactions will be given in a subsequent section of this paper.

The method for the preparation of hydrophilic colloidal sulphur was later varied in accordance with the method used by Freundlich and Scholz ('22). After the filtration through glass wool concentrated sodium chloride was added and the mixture centrifuged. The coagulum was then peptized with 100 cc. of distilled water and the insoluble sulphur centrifuged out. The peptized sulphur solution was treated 3 times with 25 cc. of saturated sodium chloride and finally peptized in 100 cc. of distilled water.

Another method for the preparation of hydrophilic colloidal sulphur was that first used by Selmi ('52) and was as follows. Sulphur dioxide was passed into distilled water until a saturated

solution was formed. Hydrogen sulphide was then passed into the sulphurous acid solution, care being taken not to have an excess of the hydrogen sulphide, as it precipitates the hydrophilic colloidal sulphur forming the hydrophobic colloid. The solution was then centrifuged to remove the larger particles and the supernatant liquid coagulated with sodium chloride. The coagulum was then peptized in water as before.

The hydrophobic colloidal sulphur can be prepared in a number of ways. It is the "milk of sulphur" formed when sulphur is precipitated out of solution. It was prepared in this work by the method used by v. Weimarn and Molyschew ('11) which was as follows: Sulphur was recrystallized in toluol and the toluol evaporated off at 60–70° C. Five-tenths gm. of this was heated with 125 cc. of fresh distilled absolute alcohol in a reflux condenser for 60 minutes. Seven cc. of this hot solution were poured into 293 cc. of distilled water at room temperature. The suspension prepared in this way was white and turbid. This was centrifuged and resuspended in water. The sulphur particles settled out of this suspension in a comparatively short time.

In determining the toxicity of these forms of colloidal sulphur the same method was used as in the preceding tests. With the hydrophilic colloidal sulphur, however, it was necessary to make a much weaker suspension. The stock colloidal suspensions contained about 1.5 per cent sulphur. Five cc. of this stock suspension were diluted to 25 cc. with distilled water, and then 1 cc. of this was added to each of the hydrogen-ion concentrations. This gave a further dilution of 1:5 and resulted in a very weak suspension of colloidal sulphur. After a preliminary test, however, the hydrophobic colloidal sulphurs were not diluted with water, and 1 cc. of the stock suspension was added directly to the culture solutions. The organisms used and the results are recorded in table 1 and figs. 1–4.

With the 6 organisms used in this experiment hydrophilic colloidal sulphur was found to be extremely toxic in the very dilute suspensions used. Only 2 of the organisms, namely, *Botrytis cinerea* and *Macrosporium sarcinaeforme,* showed a slight resistance to this suspension. In stronger suspensions germination was entirely inhibited with all the organisms used. On the other hand, hydrophobic colloidal sulphur was only slightly toxic and comparable to ground flowers of sulphur. The results indicate that sulphur is most toxic in a very finely divided state

such as is found in the hydrophilic colloidal sulphur. The influence of the hydrogen-ion concentration was very striking, especially with this latter form of sulphur. Upon examination of the culture tubes containing the hydrophilic colloidal sulphur it was found that settling out was rapidly increased as the P_H increased beyond P_H 5.4.

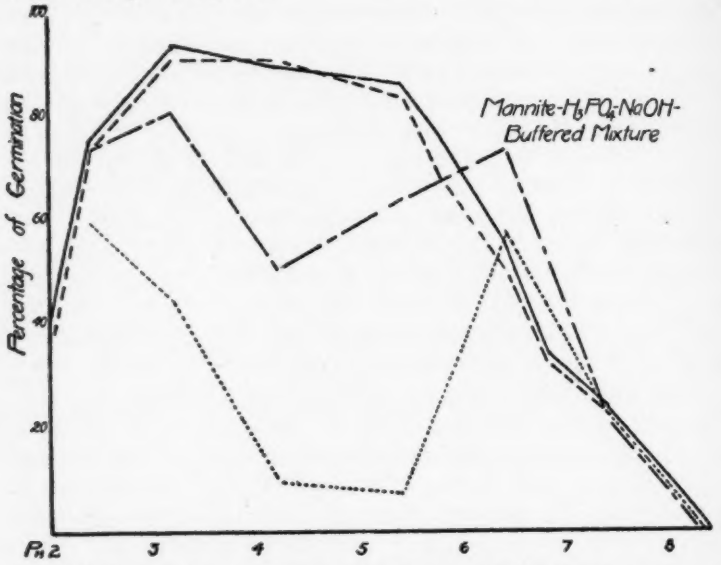

Fig. 1. Germination of spores of *Botrytis cinerea* in hanging-drop cultures: toxic action of flowers of sulphur ————; of hydrophobic colloidal sulphur - - - - - -; of hydrophilic colloidal sulphur ····· ; check, without sulphur ————.

EXPERIMENT 4. THE TOXICITY OF LIME SULPHUR

The compounds formed in lime sulphur mixtures have been fairly well determined by Haywood, Van Slyke, and others. The reactions that take place when sulphur and calcium oxide are boiled together are about as follows:

$$3Ca(OH)_2 + 12S = CaS_2O_3 + 2CaS_5 + 3H_2O$$
$$\text{or } 3Ca(OH)_2 + 8S = CaS_2O_3 + CaS_3 + 3H_2O$$

These reactions are influenced, of course, by the initial ratio of the ingredients. Varying amounts of CaS_3, CaS_4, CaS_5, CaS_2O_3 and $CaSO_3$ are formed depending upon this ratio. When lime sulphur is prepared according to the Van Slyke method, that is,

boiling together 80 lbs. of sulphur, 36 lbs. of lime, and 50 gallons of water, the first reaction is the more probable one. When prepared in this way the mixture has about the following composition: sulphur as sulphides (largely pentasulphides), 80.7 per cent, as thiosulphates, 19 per cent, as sulphites and sulphates, 0.03 per cent.

Lime sulphur mixtures are extremely alkaline and their initial efficiency as a fungicide may be due partly to this causticity, that is, to the free hydroxyl ions. An experiment was performed to determine how long this causticity remained when the spray

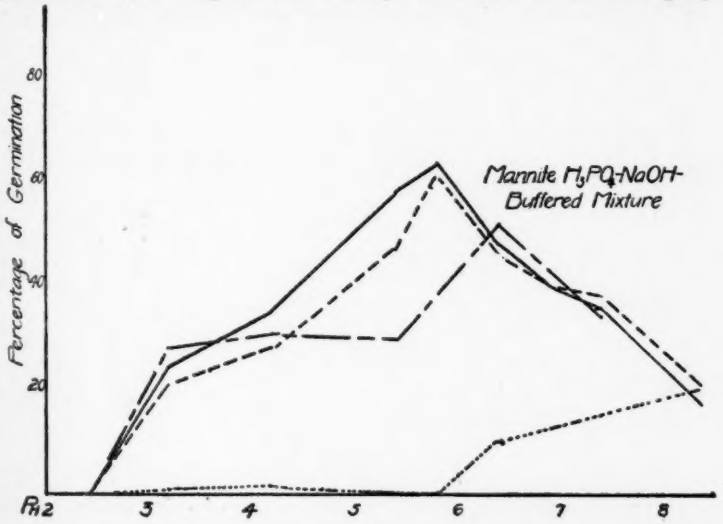

Fig. 2. Germination of spores of *Colletotrichum Gossypii* in hanging-drop cultures: toxic action of flowers of sulphur – – – –; of hydrophobic colloidal sulphur – – · – –; of hydrophilic colloidal sulphur ·····; check, without sulphur ————.

was applied, and to ascertain, if possible, whether this factor was the principal one in giving lime sulphur its prolonged effectiveness as a fungicide. For this purpose lime sulphur was prepared according to the formula given by Van Slyke, and 1 part of the lime sulphur diluted with 6 parts of water. This is a little stronger than the concentration used as a dormant spray. Twelve large moist chambers were sprayed with this mixture and kept under the following conditions: Four were exposed to dry air; a second set of 4 was placed under slightly humid conditions, and a third set of 4 in a saturated condition. After 2 hours

the lime sulphur was washed from 1 of the exposed glass dishes and the reaction determined. It was found to have changed from an initial reaction of beyond the alkaline P_H range of indicators available (P_H 10.0) to P_H 6.4. Likewise, at the end of the same length of time the mixture was washed from one of the vessels in the second set and tested for its reaction. The reaction in this case remained beyond P_H 10.

At the end of 6 hours the reactions were again determined. The wash from the first set remained the same. In the second set the reaction had changed to P_H 7.4 and in the third set it was still beyond P_H 10. At the end of 24 hours a third set of readings

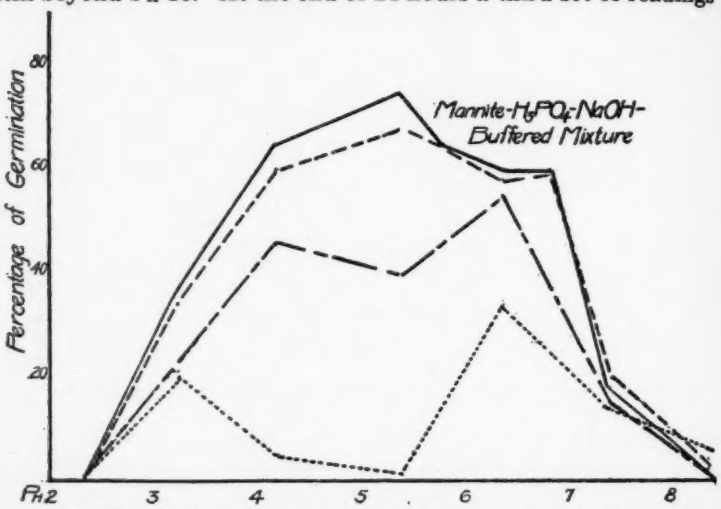

Fig. 3. Germination of spores of *Gloeosporium venetum* in hanging-drop cultures: toxic action of flowers of sulphur– – – – – –; of hydrophobic colloidal sulphur – ——— ——; of hydrophilic colloidal sulphur .....; check, without sulphur ————.

was made. All gave the same reaction, P_H 6.4. The mixture placed under the third condition did not dry out, but changed in reaction to the same point as the others. It would appear from these results that the lasting action of lime sulphur is not due to its causticity.

At this point it was thought advisable to make some chemical determinations of the exposed or changed lime sulphur. Using the same method as that listed by the Association of Official Agricultural Chemists ('20) it was found that polysulphides were absent. The percentage of thiosulphates as determined by the

Shaffer and Hartman method ('21) was 1.4. Sulphides were found to be approximately 0.1 per cent. Precipitated sulphur as determined by the carbon bisulphide method gave a percentage of 2.8. We have present then in the changed lime sulphur, precipitated sulphur, calcium thiosulphate, calcium sulphite, and calcium sulphate.

The toxicity of these individual compounds was next determined. Fifty cc. of 1:6 lime sulphur solution were set aside in a large open vessel. After 36 hours the reaction had changed to P_H 6.4. The solution was then removed and the vessel washed with

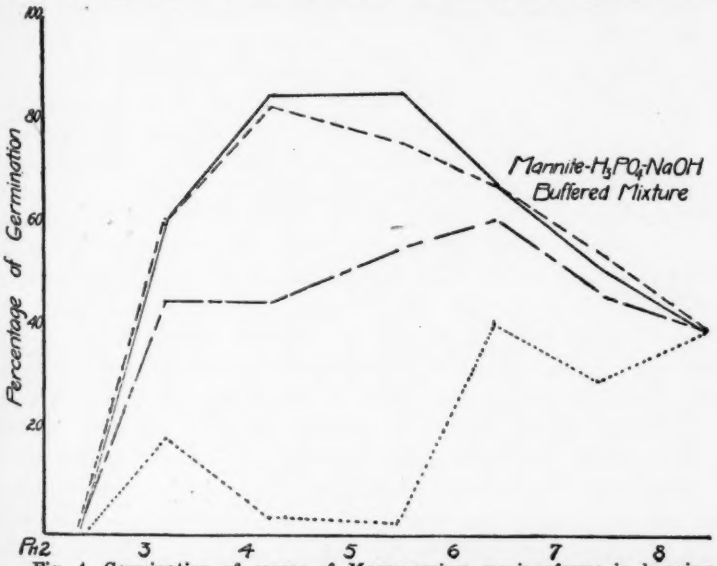

Fig. 4. Germination of spores of *Macrosporium sarcinaeforme* in hanging-drop cultures: toxic action of flowers of sulphur ——————; of hydrophobic colloidal sulphur – —— – ——; of hydrophilic colloidal sulphur ·····; check, without sulphur ——————.

sufficient distilled water to make the total quantity up to the original 50 cc. This mixture was then centrifuged until the supernatant liquid was clear. A test was then made for calcium thiosulphate in the supernatant liquid. The percentage was 1.4. The part thrown out of the solution by the centrifuge was again washed in cold water and again centrifuged. The washing removed any soluble compound that might have been present. This washed substance was then suspended in 50 cc. of distilled water. The compounds contained in this suspension were the insoluble

compounds that were formed in the changed lime sulphur, such as the sulphites and sulphates and the precipitated sulphur. The toxicity of these compounds was determined in the same way as in the preceding experiments.

The calcium thiosulphate solution did not inhibit germination of any of the organisms used. Similar results were obtained by Armstrong ('21) in his studies on sulphur nutrition of the fungi. Accordingly, calcium thiosulphate cannot be a factor, even at this high concentration, in the fungicidal value of lime sulphur.

One cc. of the precipitated sulphur suspension was placed in each of the tubes containing the slightly buffered solution. This made a further dilution of 1:5, making this final suspension equal to that of the original changed lime sulphur, that is, 1:6. The toxicity was determined in the same way as in the preceding tests. The results are given in table 1.

The results were very similar to those obtained with colloidal sulphur. The hydrogen-ion concentration influenced the toxicity in the same general way. To make sure that this toxicity was not due to the sulphites and sulphates the sulphur was coagulated out as in the case of colloidal sulphur and the toxicity again determined. The results were the same. A further test was made, using a 0.1 per cent solution of calcium sulphite, but no toxicity resulted. An attempt was next made to try to further purify the sulphur suspension by fractional centrifugation, in which the centrifuge was run very slowly, thus throwing out the sulphur and not the insoluble calcium sulphites. By repeating the centrifuging 5 or 6 times a sulphur suspension was obtained which when dried was completely soluble in carbon bisulphide. The results with reference to toxicity were the same as those cited above.

It must be concluded from these results that the lasting fungicidal value of lime sulphur is due almost entirely to the precipitated sulphur, directly or indirectly, and not to the calcium thiosulphate and the insoluble sulphites. The precipitated sulphur formed in the changed lime sulphur is not in as finely divided state as the soluble colloidal sulphur prepared by the above methods, as was shown by the slow speed with which it could be thrown out of suspension. However, its toxicity was slightly greater than that of the hydrophobic colloidal sulphur in the same concentration.

TABLE I

PERCENTAGE OF GERMINATION*

Organism	Form of Sulphur	Hydrogen-ion concentration (P_H)									
		1.6	2.4	3.2	4.2	5.4	5.8	6.4	6.8	7.4	8.4
Botrytis cinerea	Without sulphur	4	76	94	90	86	76	56	34	24	0
	Flowers of sulphur	4	74	91	91	84	68	51	33	23	0
	Ground flowers of sulphur	4	66	78	49	68	—	52	—	16	0
	Hydrophilic colloidal sulphur	6	60	45	10	8	—	58	—	23	0
	Hydrophobic colloidal sulphur	4	74	81	51	64	—	74	—	21	—
	Precipitated lime sulphur	—	60	52	20	16	—	17	22	18	—
Colletotrichum Gossypii	Without sulphur	0	0	24	35	57	64	48	40	36	18
	Flowers of sulphur	0	0	21	28	47	61	46	40	38	21
	Ground flowers of sulphur	0	0	23	25	28	—	35	—	30	—
	Hydrophilic colloidal sulphur	0	0	1	1	0	0	10	—	15	20
	Hydrophobic colloidal sulphur	0	0	28	31	30	—	52	—	34	—
	Precipitated lime sulphur	0	0	12	13	11	—	26	—	28	—
Sclerotinia cinerea	Without sulphur	0	48	72	94	82	80	76	29	3	0
	Flowers of sulphur	0	0	0	0	0	0	0	0	0	0
	Hydrophilic colloidal sulphur	0	0	0	0	0	0	0	0	0	0
Gloeosporium venetum	Without sulphur	0	4	36	65	75	65	60	60	18	2
	Flowers of sulphur	0	3	34	60	68	65	58	59	21	4
	Ground flowers of sulphur	0	3	21	46	40	—	55	—	15	3
	Hydrophilic colloidal sulphur	0	4	20	5	1	—	34	—	14	6
Macrosporium sarcinaeforme	Without sulphur	0	4	60	85	86	—	68	—	52	40
	Flowers of sulphur	0	4	61	83	76	—	68	—	55	41
	Ground flowers of sulphur	0	4	45	45	56	—	61	—	48	40
	Hydrophilic colloidal sulphur	0	0	19	3	2	—	41	—	30	38
Phomopsis Sojae	Without sulphur	0	0	1	42	31	83	—	63	37	26
	Flowers of sulphur	0	0	0	0	0	0	0	0	0	0

* Average of 6–12 replications conducted at 3 different times.

EXPERIMENT 5. THE TOXICITY OF THE VOLATILE PRODUCTS OF SULPHUR

The results of the foregoing experiments indicate that sulphur is most toxic when it is in a finely divided state, this toxicity increasing in proportion to the fineness of the particle, hydrophilic colloidal sulphur exhibiting the highest degree of toxicity. The prevailing supposition that sulphur is only toxic when in a volatile state might be justified by the assumption that finely di-

vided sulphur yields a volatile product more readily. On the other hand, the peculiar relation of this toxicity to a definite range of hydrogen-ion concentration points rather towards the probability that sulphur is toxic because of a compound produced that may be volatile, the production of this compound being affected directly by the reaction. It does not seem probable that the colloidal sulphur particle as such could be rendered non-toxic by so slight a change in reaction as has been shown to govern its fungicidal property.

To determine these points a series of experiments was arranged, using flowers of sulphur, hydrophilic and hydrophobic [1] colloidal sulphur. The organisms used were *Botrytis cinerea, Colletotrichum Gossypii,* and *Sclerotinia cinerea.* The method of procedure was the same as in the preceding experiments, with the following modifications: The spores were placed in drops of the slightly buffered solution without sulphur. The sulphur suspensions were placed only at the bottom of the cells. In this way the spores were not in direct contact with the sulphur, the distance between culture drop and cell liquid being the height of the cell, which was 8 mm. The cultures were incubated at 22° C. The results are given in table II.

TABLE II

PERCENTAGE OF GERMINATION

Organism	Form of Sulphur	Hydrogen-ion concentration (P_H)									
		1.6	2.4	3.2	4.2	5.4	5.8	6.4	6.8	7.4	8.4
Botrytis cinerea	Without sulphur	4	76	94	90	86	76	56	31	10	0
	Flowers of sulphur	4	74	91	91	84	78	51	33	—	—
	Hydrophilic colloidal sulphur	3	60	58	12	8	54	61	50	—	—
	Hydrophobic colloidal sulphur	4	71	95	84	90	74	52	32	—	—
Colletotrichum Gossypii	Without sulphur	0	0	24	38	53	55	58	46	—	—
	Flowers of sulphur	0	0	22	37	51	50	59	45	—	—
	Hydrophilic colloidal sulphur	0	0	10	0	0	21	43	47	—	—
	Hydrophobic colloidal sulphur	0	0	26	28	44	53	61	42	—	—
Sclerotinia cinerea	Without sulphur	0	51	84	95	82	70	57	9	3	0
	Flowers of sulphur	0	50	66	70	74	65	58	11	0	0
	Hydrophilic colloidal sulphur	0	0	0	0	0	3	24	14	2	0
	Hydrophobic colloidal sulphur	0	36	19	8	10	65	59	11	—	—

[1] Precipitated sulphur.

The results in this table are very similar to those recorded in table I except that the flowers of sulphur exhibited no toxic action even to *Sclerotinia cinerea* and the hydrophobic colloidal sulphur was only slightly toxic with *Botrytis cinerea* and *Colletotrichum Gossypii*. The hydrophilic colloidal sulphur exhibited the usual degree of toxicity, regardless of the fact that it was a considerable distance from the spore. Toxicity was greatest in all cases at P_H 4.0–5.5, as in the previous tests.

Having determined that the toxic substance is volatile, it was thought necessary at this point to eliminate, if possible, hydrogen sulphide, sulphur dioxide, and sulphur trioxide, as factors. For these tests *Sclerotinia cinerea* was selected because it has proved to be quite sensitive to the toxic action of sulphur. Spores were placed over a saturated solution of hydrogen sulphide in a Van Tieghem cell and the cultures were incubated at 22° C. for 24 hours. Germination was not inhibited. The experiment was repeated with *Colletotrichum Gossypii* and *Botrytis cinerea* with similar results.

No toxicity could be noted with sulphur dioxide in a concentration sufficient to kill when converted into hydrophilic colloidal sulphur by the addition of hydrogen sulphide.

Sulphuric acid inhibited growth only because of its acidity, accordingly, in proportion to acidity. Positive tests for sulphur dioxide and trioxide could not be obtained in aerated sulphur suspensions that were toxic to *Sclerotinia cinerea*. These compounds, therefore, do not contribute to the toxic properties of sulphur.

EXPERIMENT 6. THE INFLUENCE OF O_2 ON THE TOXICITY OF SULPHUR

In all of the foregoing tests the only oxygen available was that present in the air enclosed in the closed-ring cells. An experiment was conducted to determine the effect of oxygen on increasing the toxicity of flowers and precipitated sulphur. Finely ground flowers of sulphur and hydrophobic colloidal sulphur were placed in the slightly buffered mixtures in the same concentration as in Experiments 1 and 2. The Van Tieghem cells were placed in Petri dishes, the bottoms of which were lined with filter-paper in which holes somewhat larger than the glass rings were cut, so that the cells might rest on the bottoms of the Petri dishes. A large drop of the sulphur suspension was placed at

the bottom of the ring. The filter-paper was saturated with water. Spores of *Sclerotinia cinerea* were placed in a drop of the culture medium without sulphur, on a cover slip which was inverted over the cell. The cells were not sealed at the top or bottom.

In the same Petri dish sealed cells were prepared. This was done for each hydrogen-ion concentration. The Petri dishes containing the cultures were arranged in a moist chamber through which air was passed. This experiment was conducted at room temperature and the percentage of germination noted after 18 hours. The results are given in table III.

TABLE III

PERCENTAGE OF GERMINATION*
SCLEROTINIA CINEREA

P_H	Ground flowers of sulphur		Hydrophobic colloidal sulphur	
	$-O_2$	$+O_2$	$-O_2$	$+O_2$
2.4	40	38	34	24
3.2	64	49	17	10
4.2	68	31	8	0
5.4	65	24	11	0
5.8	62	46	54	32
6.4	58	49	48	44

*Average of triplicate cultures.

Another experiment was conducted, in which a weak suspension of hydrophobic colloidal sulphur which did not inhibit the germination of spores of *Colletotrichum Gossypii* at any hydrogen-ion concentration was aerated for 24 hours. Air from which the oxygen was removed with pyrogallol[1] was passed through a duplicate series. The toxicity was determined with spores of *C. Gossypii* in closed-ring cells in the same manner as in Experiment I. Likewise, a similar series was arranged, using an aerated suspension of flowers of sulphur. The cultures were incubated at 22° C. and the percentage of spore germination determined after 18 hours. The results are recorded in table IV.

The results of these tests prove conclusively that the toxic property of sulphur is due to an oxidation product and that finely divided sulphur is more readily oxidized at ordinary temperatures than the ordinary sublimed sulphur.

[1] One part pyrogallol, 5 parts NaOH, and 30 parts H_2O.

TABLE IV

PERCENTAGE OF GERMINATION
COLLETOTRICHUM GOSSYPII

P_H	Hydrophobic colloidal sulphur		Flowers of sulphur	
	$-O_2$	$+O_2$	$-O_2$	$+O_2$
2.4	0	0	0	0
3.2	26	18	22	18
4.2	42	2	51	16
5.4	56	13	60	10
5.8	60	37	68	43
6.4	66	62	54	56

EXPERIMENT 7. THE INFLUENCE OF H_2O ON THE TOXICITY OF SULPHUR

The influence of water on this volatile compound was next studied. Dry colloidal sulphur was prepared by centrifuging hydrophobic colloidal sulphur and the residue dried at room temperature. This was placed in the bottom of Van Tieghem cells. Spores of *Sclerotinia cinerea* were placed in sterile distilled water on sterile cover slips and inverted over the cell. The cell was not made air-tight, thereby not eliminating any other factor except water. Checks were arranged in which a suspension was used instead of the dry sulphur, other conditions being the same. All the cultures were placed in a moist chamber at room temperature. There resulted from this experiment no inhibition when dry sulphur was used, while the suspension gave the same amount of inhibition as reported in table III. Oxygen and water are necessary factors in the formation of the toxic volatile compound of sulphur.

Chemistry of Hydrophilic Colloidal Sulphur

The results of all the foregoing experiments point towards hydrophilic colloidal sulphur as containing the toxic substance produced by the oxidation of the ordinary forms of sublimed and precipitated sulphur. It is as toxic in closed-ring cells where little oxygen is available as in open aerated cells. The other forms of sulphur tried are toxic only when oxygen is present. Hydrophilic colloidal sulphur is toxic at 21–22° C. to *Botrytis cinerea, Macrosporium sarcinaeforme, Gloeosporium venetum,* and *Colletotrichum Gossypii,* all of which are very re-

sistant and grow normally in a suspension of flowers of sulphur at temperatures below 25° C. Because of these facts it is logical to assume that the toxic property of sulphur is due to a compound formed by the oxidation of sulphur. Having eliminated the more common oxides and acids of sulphur it was thought that this toxic compound might be one or a mixture of the more complex polythionic acids. At any rate hydrophilic colloidal sulphur contains such an acid. The chemistry of hydrophilic colloidal sulphur has been studied by a number of investigators. Bary ('20) studied Raffo's soluble sulphur (here termed hydrophilic colloidal sulphur), and came to the conclusion that the substance contained not only sulphur but polythionates. He thought the solution was made stable by the presence of small amounts of electrolytes. Freundlich and Scholz ('22) made a very extensive study of the so-called soluble sulphur and concluded that it was largely pentathionic acid. They base their conclusion on the following reactions which would take place if pentathionic acid were present.

$$2NaOH + H_2S_5O_6 = Na_2S_3O_6 + S_2 + 2H_2O$$
$$2\,Na_2S_3O_6 + 6NaOH = Na_2S_2O_3 + 4\,Na_2SO_3 + 3\,H_2O + 4S$$
or $$2Na_2S_5O_6 + 6NaOH = 5Na_2S_2O_3 + 3\,H_2O.$$

By the aid of this reaction they were able to determine qualitatively and quantitatively the pentathionic acid. The qualitative test was made by the addition of an alkali which precipitated out the sulphur in the form of a white turbid solution. They state that this test applies only to pentathionic acid, and to no other sulphur compound containing oxygen, such as any of the well-known acids or oxides. The quantitative test is made by treating the colloidal solution with normal NH_4OH, forming ammonium thiosulphate and titrating this with 0.01N iodine solution. With hydrophobic colloidal sulphur these tests were negative. They designate colloidal sulphur in this form as S λ and the form associated with pentathionic acid as S μ. When S μ is precipitated out of hydrophilic colloidal sulphur it probably becomes S λ. Such a change also takes place when pentathionic acid is treated with H_2S.

According to these workers, sodium thiosulphate and sulphuric acid react as follows:

$$Na_2S_2O_3 + H_2SO_4 = H_2S_2O_3 + Na_2SO_4$$
$$H_2S_2O_3 = SO_2 + 3S + H_2O$$

By the action of remaining sulphuric acid,

$$H_2SO_4 + 5H_2S_2O_3 = 2H_2S_5O_6 + 3\,H_2O$$

The pentathionic acid then joins with sulphur ($S\,\mu$) and water to form the hydrophilic colloid of the following structure:

$$S\,\mu$$
$$\cdot$$
$$S_5O_6\ H_2\ S_5O_6$$
$$\cdot$$
$$H_2O$$

The possibility of such a structure is based on the fact that a compound containing so many oxygen ions must necessarily have a great affinity for water. Moreover, a molecule containing so many sulphur atoms would, because of its residual valence, account for its combining with other atoms of sulphur. This being true, pentathionic acid would have the property of combining between molecules of sulphur and water. In other words, it is an adsorptive medium for both these substances. A similar phenomenon is described and illustrated by Langmuir ('17) in his studies of secondary valences in mixtures of fats and water.

Having no such adsorption medium present in hydrophobic colloidal sulphur, the $S\,\lambda$ absorbs water and forms the grouping $S\,\lambda \cdot H_2O$, which is a typical suspension colloid, poorly hydrated and gradually settling out.

The chemical nature of pentathionic acid has been very thoroughly studied. It was discovered by Wackenroder ('46) in 1845. He prepared the acid by passing H_2S into a saturated solution of SO_2, always keeping the excess of the latter. By calculations he arrived at the formula of $H_2S_5O_6$. For quantitative determinations he precipitated the acid with an alkali, in much the same way as reported by Freundlich and Scholz ('22) for hydrophilic colloidal sulphur. He also found that salts precipitated it.

After the discovery of this acid considerable controversy arose as to its existence in a pure state. Spring ('82) states that it is his opinion that the so-called pentathionic acid consists of a solution of sulphur in tetrathionic acid and that salts obtained from this solution are simply tetrathionates containing admixed sulphur. That this conclusion was partially correct was proved by Shaw ('83). He could produce pure pentathionic acid, but at times such an admixture as obtained by Spring would be obtained. A close relationship undoubtedly exists between pentathionic acid

and sulphur. Shaw prepared his pentathionic acid by passing simultaneously hydrogen sulphide and sulphur dioxide into 3 liters of distilled water for 32 hours, the sulphur dioxide being kept slightly in excess. This controversy was definitely settled by Debus ('88). His work is summed up by Mellor ('17) in the chapter on the compounds of sulphur and oxygen.

The properties of pentathionic acid have been more recently studied by Raschig ('20) and Riesenfeld and Feld ('21). The latter state that the action of H_2S and SO_2 forms the hypothetical acid $H_2S_3O_4$ as an intermediary product and that by its oxidation and reduction pentathionic acid is formed; they give the following reactions:

$$2SO_2 + H_2S = H_2S_3O_4$$

$$\left.\begin{aligned} H_2S_3O_4 + SO_2 &= H_2S_4O_6 \\ H_2S_3O_4 + 6SO_2 &= 2H_2O + H_2S_5O_6 \end{aligned}\right\} \text{Oxidation by } SO_2$$

$$H_2S_3O_4 + 3H_2S = 6S + 4H_2O\text{—Reduction by } H_2S$$

$$5H_2S_3O_4 = 3H_2S_5O_6 + H_2O\text{—Polymerization}$$

They studied the action of acid and alkali and found that the acid was unstable in both conditions.

It is therefore evident that the hydrophilic colloidal sulphur prepared according to the methods of Selmi ('52), Raffo ('08), Odén ('13), and others, is pentathionic acid. That this is an oxidation product of sulphur seems a logical conclusion. The influence of the hydrogen-ion concentration also points toward pentathionic acid as being the toxic factor in all of the preceding experiments. Flowers of sulphur, hydrophobic colloidal sulphur, and especially hydrophilic colloidal sulphur exhibited toxicity only at P_H 4.2–5.4, because of the fact that pentathionic acid is destroyed when in a solution of higher or lower hydrogen-ion concentration.

To obtain further proof of the toxicity of pentathionic acid the hydrophilic colloidal sulphur was freed of this acid. The colloidal sulphur was prepared by the following method, which is only a slight modification of the one used in previous experiments: Thirty cc. of a saturated solution of sodium thiosulphate were slowly added to 10 cc. of concentrated sulphuric acid. The mixture was warmed and filtered through glass wool. The filtrate was then coagulated with sodium chloride and centrifuged. The coagulum was peptized in 100 cc. of distilled water and again centrifuged to remove insoluble sulphur. Coagulation,

centrifuging, and peptizing were repeated 3 times, and the final coagulum peptized in 100 cc. of distilled water. The reaction of this peptized solution was P_H 4.2. Seventy-five cc. of this solution, for convenience designated No. 1, were treated with 25 cc. of normal ammonium hydroxide and let stand 24 hours, a white precipitate being formed. This was neutralized and centrifuged. The residue was suspended in 75 cc. of distilled water and designated solution 2. The filtrate, No. 3, was again treated with 25 cc. of normal ammonium hydroxide and left for 24 hours. A slight precipitate was formed. This was neutralized, the precipitated sulphur centrifuged out and suspended in 10 cc. of water, and this last designated solution 4; and the filtrate, No. 5. Seventy-five cc. of the filtrate, No. 5, were again treated with 25 cc. of normal ammonia and left for 24 hours. No precipitate formed. This was neutralized and called solution 6.

Fifty cc. of solution 2 were treated with 25 cc. of normal ammonium hydroxide and kept for 24 hours. It was then neutralized and centrifuged. The residue was suspended in 50 cc. of distilled water and designated solution 7. Twenty-five cc. of this solution were treated with 10 cc. of ammonia, allowing the usual interval, then again neutralized, centrifuged, and suspended in 25 cc. of water, constituting solution 8. A portion of each of these solutions was tested for pentathionic acid, with the result that Nos. 3, 5, 6, and 8 gave no positive test; Nos. 1, 2, and 7 gave positive tests, but 2 and 7 only a slight indication.

These solutions were then tested in respect to toxicity in closed-ring cells, using the spores of *Botrytis cinerea* and *Colletotrichum Gossypii*. The cultures were placed at 22° C. for 24 hours, and the results, which are averages of duplicate cultures, are given in table v.

TABLE V

PERCENTAGE OF GERMINATION

Organism	No. of solution								
	1	2	3	4	5	6	7	8	Ck.
Botrytis cinerea	0	10	50	41	55	55	26	54	53
Colletotrichum Gossypii	0	7	61	54	65	64	18	63	61

The amount of killing was directly proportional to the amount of pentathionic acid present. No. 8 contained as much sulphur as No. 1 but was not toxic.

Another experiment was conducted to ascertain if aerated flowers of sulphur produces pentathionic acid. Two lots of 50 gms. each of flowers of sulphur were placed in wash bottles. The 2 bottles were placed in series each connected with wash bottles containing distilled water to collect any volatile water-soluble compound that might come over. Air was passed through one series and air deprived of oxygen through the other. Aeration was continued for 72 hours. At the end of this time H_2S was passed into the distilled water wash bottles and permitted to stand for 12 hours. A slight precipitate was noted in the distilled water through which air containing oxygen had passed. The series without oxygen gave no precipitate. This afforded definite proof that pentathionic acid is an oxidation product of flowers of sulphur at ordinary temperatures. A concentrated solution of sodium chloride was added to the aerated sulphur suspensions, and centrifuged; the residue was resuspended in water and again centrifuged. Hydrogen sulphide was then passed into the supernatant liquid. A precipitate developed only in the one in which oxygen was present.

A similar series was arranged using precipitated sulphur containing no pentathionic acid. The distilled water containing the volatile soluble compound and the aerated suspension were tested for pentathionic acid. The former gave a slight precipitate with H_2S after standing. The suspension gave a much heavier precipitate indicating that the pentathionic acid was adsorbed by the sulphur particle and was not easily driven off by slow aeration. Without oxygen there was no pentathionic acid produced.

The precipitated sulphur was much more easily oxidized than the sublimed flowers of sulphur.

Practical Applications

Time has not permitted a more extensive study of this phase of the problem. It was necessary in the first place to determine the compound of sulphur that is toxic to fungi and to develop a material that would act as a fungicide over a sufficient period when sprayed on the plant. The fact that flowers of sulphur must be acted upon by some definite external physical factor has limited its use to only a small section of the country. It has been the aim in this work to develop, if possible, a sulphur compound that would exhibit fungicidal properties regardless of climatic factors and would for that reason have a wide usage over a large

part of the country. To accomplish this the material must yield readily the toxic compound, pentathionic acid. The reaction must be kept slightly acid (P_H 4.0–5.5), as this toxic compound is destroyed above or below this point. It must be readily oxidizable at ordinary temperatures. It should have great adhesiveness; it must not burn the leaves.

Colloidal sulphur has all these properties when tested in the laboratory and greenhouse. It is almost impossible to wash it from the leaves of plants after it has dried. It is difficult to remove it with a strong stream of water. Certainly rain would have little effect upon it.

That colloidal sulphur is readily oxidized has been demonstrated in the foregoing experiments. Kuhl ('21) states that colloidal sulphur bears the same relation to atmospheric oxygen as phosphoric iron, the latter being self-inflammable.

Methods for the preparation of colloidal sulphur mixtures for fungicidal use are being experimented upon. The hydrophilic colloidal sulphur prepared by the method given above is suitable as a spray. It did not burn the leaves of bean, potato, tobacco, rose, and geranium when sprayed on them. By the use of commercial materials this mixture is not too costly for practical purposes. Other methods for its preparation are being tried.

The method for the preparation of hydrophobic colloidal sulphur for trials in the greenhouse was as follows: One gallon of commercial or home-made lime sulphur was diluted with 5 gallons of water. Commercial phosphoric acid was added until the reaction was slightly acid. A milky precipitate of colloidal sulphur was formed. The mixture was allowed to stand a day or two to remove excess H_2S, and then applied. The advantage of phosphoric acid over other acids is that the calcium acid phosphate formed maintains the proper reaction. This mixture diluted 1:5 with water prevented the germination of *Botrytis cinerea* and *Colletotrichum Gossypii* in aerated cultures. When sprayed on the plant this type of colloidal sulphur does not stick as well as hydrophilic colloidal sulphur but no doubt can be made just as effective a spray by the addition of soluble glue or other suitable spreaders. Any precipitated sulphur to which has been added calcium acid phosphate or another suitable compound for maintaining the slightly acid reaction should be an effective fungicide.

With respect to increasing the value of flowers of sulphur as a spray the writer is not yet prepared to make a definite recom-

mendation. However, the fact that this substance is slowly oxidized at ordinary temperatures leads to the possibility of its being used effectively when treated with compounds that will increase its oxidation. It will also be necessary to add to such a spray an adsorptive material to retain the pentathionic acid as it is produced. Many of the common spreaders now in use may do this. These possibilities are being investigated and will be reported later.

Since the completion of the experimental part of this work there has come to my attention a number of colloidal sulphur preparations that have proved effective as a general spray. Ramsay and Cooke ('22) have prepared a colloidal sulphur that has been used effectively in Australia. They prepare their compound as follows: Ten gallons of home-made lime sulphur (26° Baumé) are diluted with 25 gallons of water in a barrel of 50 gallons capacity. In a suitable vessel 6 pints of strong commercial sulphuric acid are diluted with 9 parts of cold water and allowed to cool. The cold diluted sulphuric acid is then carefully added to the dilute lime sulphur in the barrel, a pint at a time, stirring well until the typical yellow color of the original lime sulphur disappears and until further addition of more acid produces no further precipitation of sulphur. The precipitated sulphur is allowed to settle for a day or two. Three pounds of cheap glue are dissolved in sufficient hot water to render the glue soluble and while still hot is stirred thoroughly into the sulphur. The glue aids in the keeping qualities of the colloidal sulphur. The mixture so prepared is diluted to 250 gallons (with water). This gives a spray containing approximately 5 pounds of precipitated sulphur per 100 gallons.

Thiele ('21) recommends the use of colloidal sulphur in the form of a liquid spray (not dust) for the control of mildews in Germany. He states that it is far more effective than the most finely ground sulphur powder. The colloidal mixtures adhere firmly to the plant and are not blown away by the wind or washed off by rains, as is the powder. Precipitated sulphur as a control for mildew and related fungi has been recommended by Lederle ('22). He prepared this precipitated sulphur as follows: Solution I: 250 gms. of sodium hyposulphite are dissolved in ¾ liter of hot water. Solution II: 250 gms. of sodium bisulphate are dissolved in ¾ liter of hot water. Solution III: 10 gms. of glue are dissolved in ¼ liter of hot water. Solution III is then stirred

while hot into solution I. After diluting solutions I and II each with 4 liters of water they are mixed and let stand for 3–18 hours when the mixture is ready for use. It is somewhat unstable and should be used within a few days, preferably the next morning.

Kuhl ('21) experimented with De Haen's colloidal soluble sulphur[1] and found it to be very effective in controlling mildews and related diseases. He stated that the mixture was very adhesive and that it did not burn the leaves. He believed that the increased effectiveness of this type of sulphur over other sulphur sprays was due to its increased chemical activity.

Barker and Wallace ('22) describe a new method for sulphur fumigation for the greenhouse. In previous studies they found that the fungicidal value of sulphur depended upon its being applied as extremely finely divided particles. Their method is as follows: Air is passed through molten sulphur in a Campbell's "sulphur vaporiser," the temperature of the sulphur being kept just above the melting point and well below the ignition point. The melting point of sulphur is about 115° C. and its ignition point in the air is about 260° C. The most satisfactory temperature is around 170° C. Under these conditions an abundant cloud of sulphur in the particulate condition is produced. An improvement in the yield of particulate sulphur is effected if the current of air is passed into the molten sulphur through a perforated nozzle. By means of an attached delivery tube the particulate sulphur can be discharged in any given direction and on to any definite object. It can be used for general fumigation or for direct spraying.

Another method for fumigation has been described by Vogt ('21), and is as follows: Three-hundred gms. of pure roll sulphur (stick sulphur) contained in a small iron pan is liquefied and brought to the boiling point (448° C.). There is heated at the same time in a circular copper boiler 400 gms. of water. The strongly superheated steam of the latter is forced under high pressure through the boiling sulphur which vaporizes it into small mist-like drops. These drops preserve their liquid form for several hours. They possess a high degree of adhesion not otherwise common to sulphur and do not burn the leaves. A few gms. of sulphur are enough to fill an average greenhouse with clouds of vapor which in a very short time covers all free surfaces.

[1] Manufactured by De Haen at Seelze.

A strong stream of water from the hose did not remove the sulphur from panes of glass. The method is being perfected for open-air use.

Conclusions

1. Flowers of sulphur is not sufficiently toxic to inhibit the germination of spores of *Botrytis cinerea, Colletotrichum Gossypii, Macrosporium sarcinaeforme,* and *Gloeosporium venetum* in closed-ring cells at ordinary temperatures. Spores of *Sclerotinia cinerea* and *Phomopsis Sojae* were inhibited from germination.

2. Finely ground flowers of sulphur was more toxic than the unground flowers under the same conditions but only at a hydrogen-ion concentration of P_H 4.0–5.5.

3. Methods for the preparation of hydrophilic and hydrophobic colloidal sulphur have been devised.

4. Hydrophilic colloidal sulphur was extremely toxic to all the organisms used.

5. Hydrophobic colloidal sulphur was slightly more toxic than the finely ground flowers of sulphur.

6. The chemical and fungicidal properties of lime sulphur were studied. Before application lime sulphur contains 80.7 per cent sulphur as calcium sulphides, 19 per cent as calcium thiosulphate, and .03 per cent as sulphites and sulphates. After exposure to the air for a few hours as a spray the sulphides disappear and increasing amounts of sulphur are formed. The lasting effectiveness of the mixture is due to the precipitated sulphur which is about as toxic as hydrophobic colloidal sulphur.

7. The toxic property of sulphur is not due to SO_2, SO_3 or H_2S, or any of the common acids or oxides of sulphur, or to the sulphur particle. Germ tubes grew normally in a heavy suspension of precipitated sulphur in closed-ring cells.

8. The toxic property of sulphur is only exhibited when oxygen and water are present.

9. By chemical analysis the toxic property of sulphur has been found to be pentathionic acid which is an oxidation compound formed from sulphur and water.

10. Pentathionic acid is volatile and is an active adsorption compound. It is destroyed in acid and alkaline solutions.

11. Finely divided sulphur is more readily oxidized to pentathionic acid at ordinary temperatures than is the flowers of sulphur.

12. Finely divided sulphur has been used as a spray in England, Australia, and Germany, with excellent results.

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GENERAL INDEX TO VOLUME IX

New scientific names of plants and the final members of new combinations are printed in **bold face** type; synonyms and page numbers having reference to figures and plates, in *italic;* and previously published scientific names and all other matter, in ordinary type.

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